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Apoptotic effects of imidazo[1,2-*a*]pyrazine derivatives in the human Dami cell line

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Abstract

cAMP-elevating agents like phosphodiesterase inhibitors and purines have been shown to induce apoptosis. In the present work we have studied the effects of imidazo[1,2-*a*]pyrazine derivatives with a purine-like structure: PAB13 (6-bromo-8-(methylamino)imidazo[1,2-*a*]pyrazine), PAB15 (6-bromo-8-(ethylamino)imidazo[1,2-*a*]pyrazine), PAB23 (3-bromo-8-(methylamino)imidazo[1,2-*a*]pyrazine) on the growth of the Dami cell line in comparison to that of adenosine. The growth effect of PAB13, PAB15 and PAB23 was investigated in relation to their phosphodiesterase-inhibitory action and their activity on purinoceptors. Inhibition in cell growth was up to 71.0%, 76.3% and 89.7% for PAB23, PAB13 and PAB15, respectively and 100% for adenosine. Cell viability was affected in a concentration-dependent manner by PAB13, PAB15 and adenosine, with a correlation between growth inhibition and cytotoxicity. These effects of imidazo[1,2-*a*]pyrazine derivatives were found to be unrelated to an action on purinoceptors, but rather appear quantitatively linked to their ability in inducing apoptosis through their cAMP-increasing and phosphodiesterase-inhibitory potency.

Keywords: Apoptosis; Imidazo[1,2-*a*]pyrazine derivative; Megakaryoblastic cell, human; Phosphodiesterase; cAMP; Purinoceptor

1. Introduction

Apoptosis, a type of programmed cell death, is an energy-dependent process in which a cell participates in its own destruction (Duvall and Wyllie, 1986). This process is characterized morphologically by cell shrinkage, nuclear condensation, membrane blebbing, nuclear fragmentation with formation of membrane-bound apoptotic bodies (Duvall and Wyllie, 1986; Ueda and Shah, 1994; Binder and Hiddemann, 1994). Frequently, apoptosis is accompanied by cleavage of DNA into integer multiples of nucleosomal-sized fragments (Duvall and Wyllie, 1986; Ueda and Shah, 1994). Some physiological and pathological states like haematopoiesis and cancer cell growth have been associated with this process (Ueda and Shah, 1994), and the cytotoxic effect of anticancer agents like nucleosides or purine analogues has been shown to involve apoptotic cell death (Smets, 1994; Sen and D'Incalci, 1992).

There is evidence for the implication of the cAMP pathway in the transduction of the death signal (Binder and Hiddemann, 1994; Mühl et al., 1996). cAMP analogues as well as cAMP-elevating agents like cholera toxin, β_2-adrenoceptor agonists, prostaglandins, forskolin and phosphodiesterase inhibitors have been shown to induce apoptosis in different cell lines including leukaemia cells (Duprez et al., 1993; Vintermyr et al., 1993, 1995; Aharoni et al., 1995; Mentz et al., 1995; Boe et al., 1995; Mühl et al., 1996). Recently, the purine analogue and phosphodiesterase inhibitor theophylline has been shown to cause apoptosis in B lymphocytic leukaemia cells in vitro, an effect partially antagonized by a cAMP antagonist (Mentz et al., 1995).

We have synthesized a series of three imidazo[1,2-*a*]pyrazine derivatives with a purine-like structure (Bonnet et al., 1992). They have previously been reported to elicit different pharmacological properties (Bonnet et al., 1992; Michel et al., 1995; Zurbonsen et al., 1994) including a phosphodiesterase-inhibitory effect (Bonnet et al., 1992; Michel et al., 1995; Laurent et al., 1996; Pocock and

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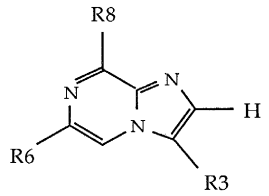

	R3	R6	R8
PAB13	H	Br	$NHCH_3$
PAB15	H	Br	NHC_2H_5
PAB23	Br	H	$NHCH_3$

Fig. 1. Chemical structure of the imidazo[1,2-*a*]pyrazine derivatives.

Small, 1996). In the present study we have investigated the effects of these compounds (Fig. 1) on the proliferation of the human megakaryoblastic leukaemic cell line Dami (Greenberg et al., 1988) which was found negatively regulated by cAMP (Vittet et al., 1995) and which bears P_2 purinoceptors (Savi et al., 1994). Since these derivatives were shown to exert antiproliferative and cytotoxic effects, we examined the possibility that they could exert these actions through an apoptotic mechanism and in relation with their effects on cAMP levels, on phosphodiesterase isoenzymes and on P_1 and P_2 purinoceptors.

2. Materials and methods

2.1. Materials

Dami cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Horse serum was purchased from Techgen International (France), and cell culture medium and reagents were from Gibco (Corning, NY, USA).

Dulbecco's phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} was obtained from Gibco BRL (France) and the P_2 purinoceptor antagonist (Lambrecht et al., 1992) pyridoxal-phosphate-6-azophenyl-2′4′-disulfonic acid tetrasodium (PPADS), the P_1 purinoceptor antagonist (Bruns et al., 1986) 8-*p*-sulfophenyltheophylline (8-pSPT), the adenosine A_1 receptor agonist (Bruns et al., 1986) N^6-cyclopentyladenosine (CPA) and the adenosine A_1/A_2 receptor agonist (Bruns et al., 1986) 5′-(*N*-cyclopropyl)-carboxamidoadenosine (CPCA) from RBI (Bioblock, France). The cAMP ^{125}I-radioimmunoassay kit was from Immunotech (France), the CytoTox 96 kit from Promega (France) and the ApopTag kit from Oncor (Appligène, France). The imidazo[1,2-*a*]pyrazine derivatives (Fig. 1), PAB13 (6-bromo-8-(methylamino)imidazo[1,2-*a*]pyrazine), PAB15 (6-bromo-8-(ethylamino)imidazo[1,2-*a*]pyrazine), PAB23 (3-bromo-8-(methylamino)imidazo[1,2-*a*]pyrazine) were synthesised as previously described (Bonnet et al., 1992).

2.2. Cells and cell culture conditions

Dami cells were grown in suspension in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum, 2 mM glutamine, 1 mM sodium pyruvate and 0.4% non-essential amino acids. They were incubated in disposable sterile Erlenmeyer flasks (Corning) at 37°C with a humidified atmosphere of 5% CO_2. Cultures were fed every 2–3 days by partial replacement of spent medium.

2.3. Proliferation studies

Cellular proliferation was studied by placing cells in the exponential phase of growth in microwells (Nunc) at a starting concentration of 2×10^5 cells/ml. Culture conditions were as described above. Cells were incubated with the products to be tested for 48 h and cell proliferation was determined by cell count performed in duplicate using a Coulter Counter ZM (Coultronics, France) equipped with a 140 μm orifice tube, and calibrated with 14 μm latex particles. An appropiate threshold of 10 μM was set to exclude cell debris.

2.4. Cell viability

Cell viability was determined by the use of the CytoTox 96 nonradioactive assay which allows the quantitative spectrophotometric measurement of lactate dehydrogenase (LDH) activity (Decker and Lohmann-Matthes, 1988). Cells were seeded and incubated with the compounds for different times (6, 12, 18, 24 and 48 h) as described by Vittet et al. (1995).

2.5. Cyclic AMP assays

Samples for determination of intracellular cAMP concentrations by a radioimmunoassay kit were prepared as follows:

Cells were washed with PBS (158 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 5.55 mM glucose, pH 7.4), resuspended to a final concentration of 2.0×10^6 cells/ml and preincubated for 5 min at 30°C before the addition of compounds to be tested. Incubations were performed at 30°C for 10 min and terminated by adding 60% cold trichloroacetic acid to a final concentration of 10%. After mixing, each sample was spun at $14\,000 \times g$ for 2 min and the supernatants were neutralised with KOH (3 mM) and buffered with HEPES (150 mM). The suspension was kept in an ice bath for 10 min and the precipitate was removed by centrifugation at $2500 \times g$ for 5 min. The neutralised extracts were stored at −20°C until assayed according to the manufacturers' recommendations.

2.6. Analysis of DNA fragmentation by agarose electrophoresis

After incubation in plastic culture flasks (T175, Falcon) with the products to be tested, cells were harvested, washed

twice with PBS (Ca^{2+} and Mg^{2+} free, Gibco) and resuspended (2×10^7 cells/ml) in a cell lysis buffer (10 mM Tris-HCl buffer, pH 8.0, and 2 mM ethylenediaminetetracetic acid) containing 1% Triton X-100 and then mixed vigorously for 1 min. After 30 min at 0°C, the cell lysate was separated into supernatant (soluble DNA) and precipitate (high-molecular DNA) fractions by centrifugation ($20\,000 \times g$, 20 min). The precipitate was redissolved with lysis buffer. Both fractions were incubated at 50°C for 4 h in the presence of sodium dodecylsulfate and proteinase K at a final concentration of 0.5% and 0.2 mg/ml respectively. After the addition of pancreatic RNase in a final concentration of 0.25 mg/ml the fractions were further incubated for 2 h. DNA was extracted twice with phenol and twice with chloroform/isoamylalcohol (24:1). To precipitate the DNA, the final aqueous phases were mixed with 2.5 vols. of cold ethanol and NaCl (0.1 M final) and stored at −20°C overnight. DNA was recovered by centrifugation at $12\,000 \times g$ for 25 min. Pellets were redissolved in H_2O. The DNA samples were electrophoresed on 1.5% agarose (Bioprobe) gel at a constant voltage of 50 V for 1.5 h (low-molecular, fragmented DNA) or of 20 V for 5 h (high-molecular DNA) and visualised by ethidium bromide staining. A molecular weight marker (No. VI, Boehringer) was run on the gel to provide standards of 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234, 220 and 154 bp.

2.7. Nuclear staining

For analysis of nuclear morphology 1×10^6 cells were pelleted and stained by resuspension in 10 μl of Hoechst 33258 solution (100 μM in PBS). After 30 min incubation, cells were examined with a Zeiss Axiophot photomicroscope equipped with an epifluorescence attachment.

2.8. Quantification of DNA fragmentation

The extent of apoptosis was quantified using a ApopTag kit (Oncor). After 24 h incubation cells were pelleted, washed twice in RPMI 1640 (without phenol red) and fixed for 15 min in ice-cold PBS (pH 7.4) containing 1% paraformaldehyde. Cells were labelled by indirect immunoflurescence using as first antibody a digoxigenin nucleotide which was catalytically added to the DNA by terminal deoxynucleotidyl transferase, and as second antibody an anti-digoxigenin fluorescein according to the manufacturer's recommendation. Flow cytometry was carried out by analysing 10 000 cells/test using a FACScan (Becton Dickinson) equipped with an argon ion laser, 15 mW and a multiparameter data acquisition system (LYSIS).

2.9. Phosphodiesterase assay

Phosphodiesterase isoenzymes were separated from Dami cells by anion exchange chromatography of the cytosolic fraction on an *O*-(diethylaminoethyl)-Sepharose CL-6B column. Three types of phosphodiesterase isoenzymes were eluted using a step-by-step Na-acetate gradient and were characterised by the selective PDE inhibitors trequinsin, rolipram and zaprinast as PDE type III, IV and V, respectively. The phosphodiesterase activity was determined by the method of Thompson et al. (1979) modified by Cook et al. (1995). Assays were performed at 37°C in a total volume of 100 μl. Each tube contained 25 μl of each fraction, 50 μl of assay buffer (final concentration of 40 mM Tris-HCl, 2.5 mM $MgCl_2$, 3.75 mM β-mercaptoethanol, 0.2 μCi [^{3}H]cAMP or [^{3}H]cGMP, 1 μM cAMP or cGMP, pH 8.0) and 25 μl test compound or its solvent. Following 30 min of incubation, the reaction was stopped by transferring to a bath of boiling water for 3 min. After cooling on ice, 20 μl of 1 mg/ml *Ophiophagus hannah* venom was added and the reaction mixture was incubated at 37°C for 10 min. Unreacted [^{3}H]cAMP or [^{3}H]cGMP was removed by the addition of 400 μl of a 35% suspension of Dowex 1x8-400 μl resin and incubation on ice for 30 min. After centrifugation ($2500 \times g$, 5 min) 200 μl of the supernatant was removed for liquid scintillation counting. Less than 10% of the tritiated cyclic nucleotide was hydrolysed in that assay.

The IC_{50} values of the compounds examined were determined from concentration-response curves. At least two concentration-response curves were generated for each agent.

2.10. Statistical evaluation

Results are means ± S.E.M. Statistical differences were determined by Student's *t*-test or Whitney *U*-test (cAMP assay); $P < 0.05$ was considered as significant. IC_{50} values were calculated using computer software (Tallarida and Murray, 1981).

3. Results

3.1. Effects of the imidazo[1,2-a]pyrazine derivatives and adenosine on Dami cell growth

All imidazo[1,2-*a*]pyrazine derivatives and adenosine inhibited Dami cell growth in a concentration-dependent

Table 1
Effects of the imidazo[1,2-*a*]pyrazine derivatives on cell growth inhibition (G.I.) and on phosphodiesterase isoenzyme activity

Compounds [b]	$-\log(IC_{50})$ [a]			
	G.I.	Type III	Type IV	Type V
PAB13	4.5 ± 0.0	4.7 ± 0.2	4.7 ± 0.1	< 4.0
PAB15	4.7 ± 0.0	4.3 ± 0.1	< 4.0	< 4.0
PAB23	4.2 ± 0.1	< 4.0	< 4.0	< 4.0

[a] Values were the means ± S.E. of 6–13 experiments performed in triplicate. [b] Compounds were assayed with 1 μM cAMP (types III and IV) or cGMP (type V) as substrate on isoenzymes isolated from Dami cells.

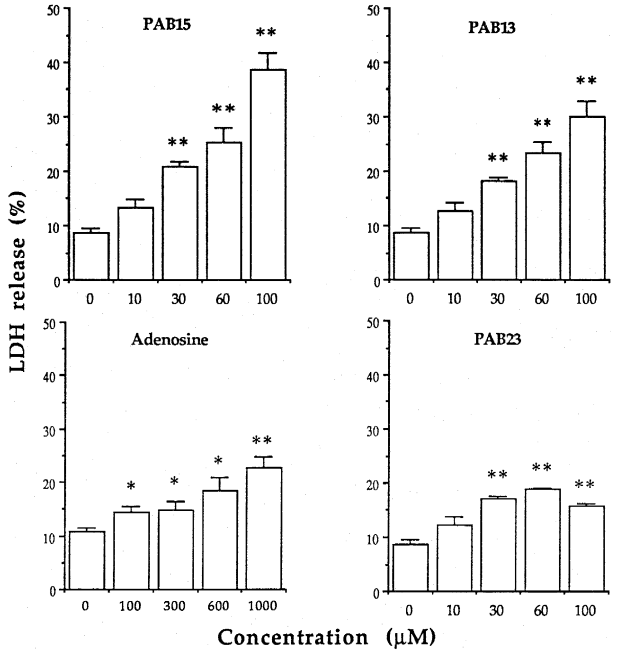

Fig. 2. Effects of 48 h exposition with imidazo[1,2-*a*]pyrazine derivatives (A) PAB13, PAB15, PAB23 and adenosine (B) on LDH release from Dami cells. Each bar represents mean ± S.E.M. of at least four experiments. Significantly different from control: * $P < 0.05$, ** $P < 0.01$.

manner (Table 1) up to 71.0%, 76.3% and 89.7% at the highest concentration for PAB23, PAB13 and PAB15, respectively and 100% for adenosine (1 mM). The $-\log(IC_{50})$ for adenosine (4.2 ± 0.1) was similar to those of the imidazo[1,2-*a*]pyrazine derivatives. Because of the poor solubility of the imidazo[1,2-*a*]pyrazine derivatives the highest concentration (100 μM) tested could not be exceeded.

3.2. Determination of cytotoxicity

48 h treatment by the different compounds induced a concentration-dependent increase of LDH release from Dami cells (Fig. 2). The cytotoxic effects of PAB13 and PAB15 were more pronounced than those induced by adenosine and PAB23. For PAB13 and PAB15 (0–100 μM) and for PAB23 (0–60 μM) a good exponential correlation ($r = 0.99$ for PAB13, PAB15 and PAB23, $r = 0.93$ for adenosine) between LDH release and inhibition of cell proliferation was observed.

Kinetic analysis of LDH release induced by the imidazo[1,2-*a*]pyrazine derivatives after 6, 12, 18 and 24 h incubation at the highest concentration (100 μM) indicated that PAB15 induced an increase in LDH release from 6 to 24 h, PAB13 elicited a significant effect only after 18 h and PAB23 presented a weak but significant effect at 6 h and a more pronounced effect at 24 h (Fig. 3).

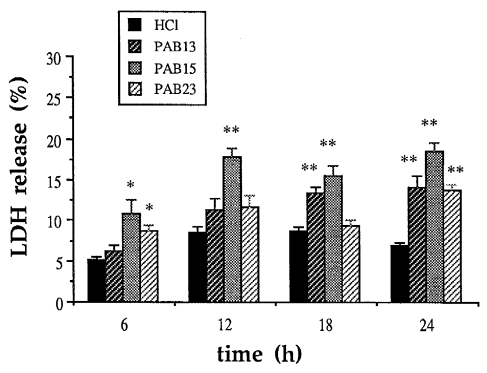

Fig. 3. Effects of 100 μM imidazo[1,2-*a*]pyrazine derivatives on LDH release after 6, 12, 18 and 24 h incubation. Each bar represents mean ± S.E.M. of three experiments. Significantly different from control: * $P < 0.05$, ** $P < 0.01$.

3.3. Effect of imidazo[1,2-a]pyrazine derivatives and adenosine on DNA fragmentation

After 24 h incubation of Dami cells in the presence of 100 μM PAB13, PAB15 and to a lesser extent with 1 mM

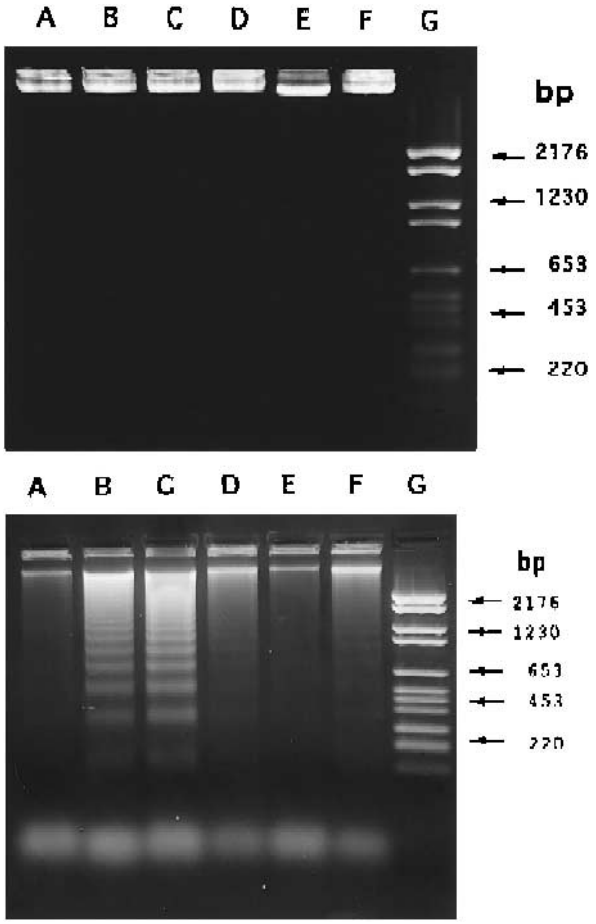

Fig. 4. Separation of soluble DNA from Dami cells incubated for 24 h with PAB13, PAB15 and PAB23 (100 μM) or adenosine (1 mM). High-molecular weight DNA (upper panel) and low-molecular weight (lower panel) fragmented DNA obtained from equal aliquots of cells was isolated and resolved by agarose gel electrophoresis as described in Section 2. Lane A, control of the PAB compounds; lane B, PAB13; lane C, PAB15; lane D, PAB23; lane E, control of adenosine; lane F, adenosine; lane G, molecular mass markers.

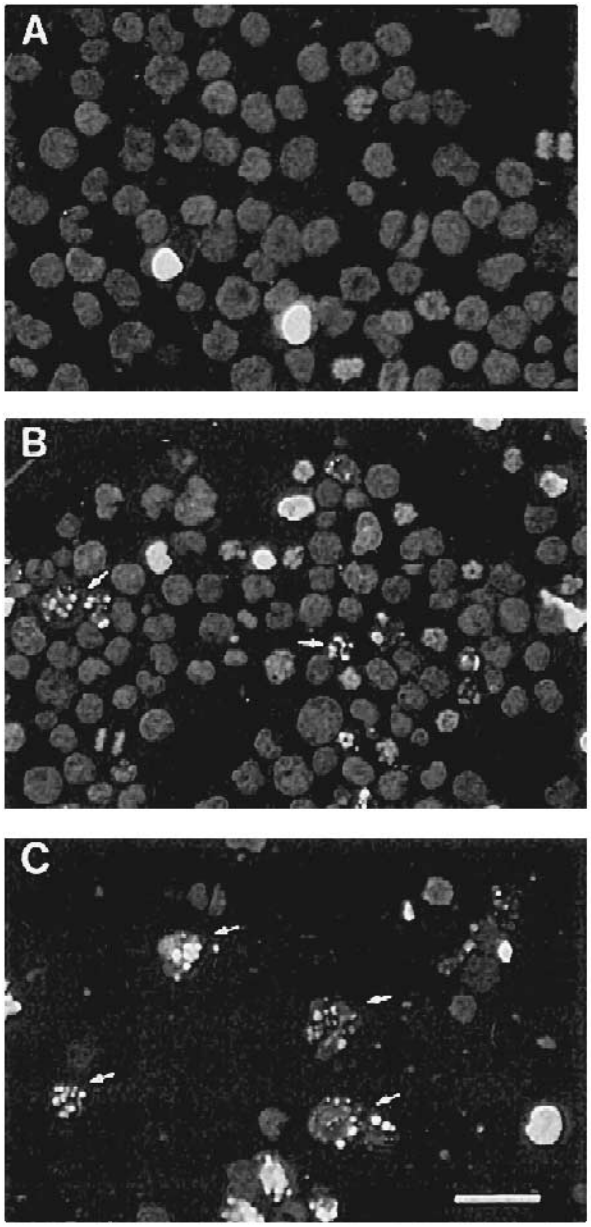

Fig. 5. Fluorescence micrographs of Dami cells treated with PAB15 (100 μM) and its control. After 24 h of exposure, 1×10^6 cells were stained with 0.1 mM Hoechst 33252. Control (A), PAB15 (B,C), apoptotic bodies (arrows). Scale bar = 40 μM.

adenosine, we could detect by DNA agarose gel electrophoresis typical DNA fragmentation with fragments of multiples of 180–200 bp. The effect of PAB23 was only marginal (Fig. 4). For PAB13 and PAB15 a weak effect could already be observed after 12 h of incubation (results not shown).

Quantification of the extent of apoptosis induced by the imidazo[1,2-*a*]pyrazine derivatives by labelling the cellular free 3′-OH DNA ends, indicated again that PAB13 and PAB15 were the most potent compounds in increasing apoptotic cell death. DNA fragmentation was 650% and 747% of untreated control cells for PAB13 and PAB15 respectively, compared to 352% for PAB23.

Table 2
Effects of imidazo[1,2-*a*]pyrazine derivatives, ADP and adenosine on Dami cell growth

Compound	G.I. (%) [a]	G.I. (%) [a] in the presence of 100 μM PPADS
PPADS [c]	–	1.2 ± 0.5
ADP [d]	41.4 ± 0.9 [b]	1.8 ± 0.9
PAB 13 [c]	76.3 ± 0.3 [b]	77.2 ± 0.9 [b]
PAB 15 [c]	89.7 ± 1.5 [b]	89.6 ± 0.5 [b]
PAB 23 [c]	66.5 ± 1.6 [b]	64.3 ± 1.2 [b]
Adenosine [e]	93.4 ± 0.2 [b]	92.6 ± 0.4 [b]

[a] Growth inhibition (G.I.) after 48 h; values are the means ± S.E. of 4–6 individual experiments. [b] Significantly different from control, $P < 0.01$ (Student's *t*-test). [c] 100 μM. [d] 500 μM. [e] 1 mM.

As shown for PAB15, DNA fragmentation as well as typical apoptotic bodies could also be observed by nuclear staining with the dye Hoechst 33258 (Fig. 5).

3.4. Effects of the imidazo[1,2-a]pyrazine derivatives and adenosine on P_1 and P_2 purinoceptors

In relation to the purine-like structure of the derivatives, we investigated whether the antiproliferative effects were induced through activation of P_1 and P_2 purinoceptors. The antiproliferative effect of adenosine was unaffected by 8-pSPT (at 10 and 100 μM). Since the weak antiproliferative effect of CPA (28% growth inhibition at 100 μM) was not antagonised by 8-pSPT and since CPCA did not present any effect on cell growth, the effect of 8-pSPT on cell growth inhibition induced by the imidazo[1,2-*a*]pyrazine derivatives was not evaluated. The CPCA could not be used at concentrations higher than 10 μM due to the poor solubility of the compound. In contrast, ADP was shown to inhibit Dami cell growth up to 42.4 ± 0.9% at 500 μM (Table 2) and this effect could be fully antagonised by PPADS whereas both imidazo[1,2-*a*]pyrazine derivatives and adenosine effects on cell proliferation remained unchanged in the presence of PPADS (Table 2).

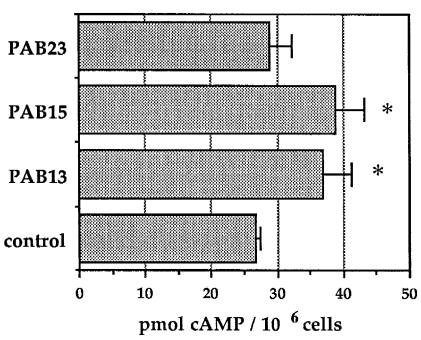

Fig. 6. Effects of 100 μM imidazo[1,2-*a*]pyrazine derivatives after 10 min incubation on intracellular cAMP levels. Histograms are the means and horizontal bars indicate the S.E.M. of 3–5 experiments performed in duplicate. Significantly different from control: * $P < 0.01$ (Whitney *U*-test).

3.5. Effects of imidazo[1,2-a]pyrazine derivatives on intracellular cAMP levels

To look for a potential relationship between cell growth inhibition, cytotoxicity and intracellular cAMP concentrations we have investigated the effect of the compounds on cAMP concentration. After 10 min of incubation PAB13 and PAB15 but not PAB23 significantly increased cAMP concentration (Fig. 6).

3.6. Effects of the imidazo[1,2-a]pyrazine derivatives on phosphodiesterase isoenzymes isolated from Dami cells

All compounds exhibited weak phosphodiesterase-inhibitory effects on the three isoenzymes isolated from Dami cells (Table 1). Only PAB15 and PAB13 presented IC_{50} values higher than 100 μM towards phosphodiesterase isoenzymes type III (PAB15) or types III and IV (PAB13).

4. Discussion

In the present study, all imidazo[1,2-*a*]pyrazine derivatives and adenosine elicited similar concentration-dependent inhibitory effects on Dami cell growth. They also induced concentration-dependent cytotoxic effects on these cells which appear to be related to their antiproliferative effects. PAB15 and PAB13 were more potent than PAB23 and adenosine in inducing cytotoxicity. Cytotoxic effects have already been shown for cAMP-elevating agents (Vintermyr et al., 1993, 1995; Aharoni et al., 1995; Mentz et al., 1995, ; Boe et al., 1995; Mühl et al., 1996; Duprez et al., 1993) and for purine and nucleoside derivatives like adenosine (Smets, 1994; Sen and D'Incalci, 1992) studied in different cell types. These effects have been related to an apoptotic mechanism. In view of the purine-like structure of the imidazo[1,2-*a*]pyrazine derivatives and their previously reported cAMP-phosphodiesterase-inhibitory effects (Bonnet et al., 1992; Michel et al., 1995; Laurent et al., 1996; Pocock and Small, 1996) we were interested to know whether they could induce cytotoxicity through such a mechanism. Since two hallmarks of apoptosis are internucleosomal DNA cleavage and morphological changes (Duvall and Wyllie, 1986; Ueda and Shah, 1994), we looked for these features. PAB13 and PAB15 clearly induced DNA double-strand cleavage with fragments of multiples of 180–200 bp which fits well with their high cytotoxic effect. As shown for PAB15, the internucleosomal DNA cleavage occurred together with morphological changes with the formation of apoptotic bodies. In contrast, the lower cytotoxicity of PAB23 and adenosine was associated with a weak induction of DNA cleavage. Thus a relationship between the cytotoxic effect of the imidazo[1,2-*a*]pyrazine derivatives and adenosine and their ability to induce apoptosis appeared.

To distinguish apoptotic from cytotoxic actions of the imidazo[1,2-*a*]pyrazine derivatives we have realised a kinetic analysis of LDH release at times preceding DNA fragmentation and morphological changes. The results indicated a cytotoxic effect of PAB15 already at 6 h, while morphological changes seen by both phase-contrast microscopy and DNA fragmentation could be observed at 12 h (results not shown). Thus the PAB15-induced LDH release at 6 h seemed preferentially due to its cytotoxic effect. However, from 12 h to 48 h the induction of LDH release could result either from a cytotoxic action of PAB15 or a secondary necrosis of apoptotic cells. Thus it appears difficult to clearly separate the apoptotic and cytotoxic actions of PAB15. In contrast, PAB13 elicited a significant effect on LDH release later, at 18 h, whereas DNA fragmentation could already be observed at 12 h by gel electrophoresis. This observation suggests that PAB13 selectively increased apoptosis without inducing cytotoxicity and that the increase in LDH release observed at 18, 24 and 48 h results from a secondary necrosis of apoptotic cells. PAB23 did not induce any significant LDH release at 12 and 18 h, which suggests that its weak cytotoxic effect at 6 h was related to cell death by necrosis due to the first contact with the compound.

Since the apoptosis-inducing effect of adenosine has been related to an activation of purinoceptors (Kizaki et al., 1990) and those of theophylline and 3-isobutyl-1-methylxanthine (Mentz et al., 1995; Duprez et al., 1993) to their cAMP-elevating potency, we studied whether the purine-like structure of PAB13, PAB15 and PAB23 and/or their phosphodiesterase-inhibitory potency were implicated in their cytotoxic and apoptotic actions.

In the Dami cell line, it appears that activation of P_1 purinoceptors did not yield to inhibition of cell growth. Indeed, selective adenosine A_1 or A_2 receptor agonists presented only a weak (A_1) or no (A_2) effect on cell growth. Furthermore, the antiproliferative effect of adenosine was not affected by the non-selective P_1 purinoceptor antagonist, 8-pSPT, which suggests that adenosine acts at the intracellular level as previously shown for the effect of adenosine on HL-60 cells (Tanaka et al., 1994). In contrast, ADP inhibited Dami cell growth, an effect that can be antagonised by the P_2 purinoceptor antagonist PPADS, whereas the antiproliferative effects of PAB13, PAB15, PAB23 and adenosine remained unchanged in the presence of PPADS. Taken together, these results rule out the involvement of P_1 or P_2 purinoceptor activation in the antiproliferative activity of the imidazo[1,2-*a*]pyrazine derivatives.

The cytotoxic effects of the imidazo[1,2-*a*]pyrazine derivatives actually appear to be related to their cAMP-elevating potency and their phosphodiesterase-inhibitory effect. Indeed, the potent cytotoxic effect of PAB13 and PAB15 was associated with significant increase in cAMP levels and inhibitory action on phosphodiesterase type III and IV isoenzymes. Conversely, the relatively low cyto-

toxic effect of PAB23 was concomitant with a lack of effect on cAMP levels and minor inhibitory effect on phosphodiesterases. These results fit well with the recent observation that both cAMP analogues and stimulators of adenylyl cyclase exerted a cell growth inhibition and/or a cytotoxic effect on the Dami cell line (Vittet et al., 1995). Furthermore, these results are in agreement with the apoptotic effects induced by the phosphodiesterase inhibitors theophylline and 3-isobutyl-1-methylxanthine (Mentz et al., 1995; Duprez et al., 1993), or those elicited by cAMP-elevating agents (Lanotte et al., 1991; Vintermyr et al., 1993, 1995; Aharoni et al., 1995; Boe et al., 1995; Mühl et al., 1996).

This study shows for the first time the ability to induce cytotoxic effects related to apoptosis in the human megakaryoblastic Dami cell line by agents which exhibit purine-like structure and phosphodiesterase-inhibitory properties. Whereas an action of the imidazo[1,2-*a*]pyrazine derivatives on P_1 or P_2 purinoceptors does not seem to be implicated in these effects, the cAMP-elevating/phosphodiesterase-inhibitory potency of the compounds appears to be related to their effects on cell growth.

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