Biochemical Pharmacology, Vol. 54, pp. 365–371, 1997.

ELSEVIER

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(97)00224-4

Antiproliferative Effects of Imidazo[1,2-*a*]pyrazine Derivatives on the Dami Cell Line

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ABSTRACT. Since cyclic 3′,5′-adenosine monophosphate (cAMP) is involved in cell proliferation and as previous data showed that imidazo[1,2-α]pyrazine derivatives (PAB12, PAB30, PAB40, SCA40, SCA41, and SCA44) inhibited cAMP breakdown by a phosphodiesterase (PDE)-inhibitory effect, the aim of the present study was to investigate the effects of these derivatives on proliferation of the Dami cell line in relation with their actions on cAMP content and on PDE isoenzymes isolated from Dami cells. SCA41 and SCA44 inhibited cell growth in a dose-dependent manner, while SCA40 and PAB40 induced a weak inhibition. Growth inhibitions were 40%, 91%, and 60% for SCA41, SCA44 (at 100 μM), and IBMX (at 1000 μM), respectively, and could not be related to their effects on cAMP levels. In addition, although all compounds potentiated cAMP formation by prostaglandin E1 (PGE1), no potentiations were observed when the antiproliferative effects of SCA41 and SCA44 were considered. Investigation of derivatives on PDE isoenzymes III, IV, and V indicated non-selective PDE inhibitory effects for SCA41 and SCA44, while SCA40 elicited preferences for type III, and PAB30 and PAB40 preferences for type IV isoenzymes. These effects could not totally explain the antiproliferative activity of the derivatives. The activation of P2 purinoceptors by imidazo[1,2-*a*]pyrazine did not lead to their antiproliferative effects. Thus, the mechanism of the antiproliferative effects of the compounds remains to be determined. It does, however, depend on the chemical substitutions of the imidazo[1,2-*a*]pyrazine skeleton and in particular on the 2-carbonitrile presence and the length of the 8-aminoaliphatic group. BIOCHEM PHARMACOL 54;3:365–371, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. proliferation; cAMP; imidazo[1,2-*a*]pyrazines; PDE isoenzymes; purinoceptors; megakaryoblastes

Cyclic 3′,5′-adenosine monophosphate (cAMP) is involved in cell proliferation and differentiation [1–3]. Intracellular cAMP concentration may be modulated by an effect on its synthetic pathway, i.e. stimulation of receptors by extracellular signals that activate or inhibit adenylyl cyclase, or through an effect on phosphodiesterase (PDE)[‖], the enzymes which destroy cyclic nucleotides.

The cAMP pathway has been considered to be a potential target for the development of new anticancer drugs [4]. In particular, cAMP analogs [3, 5, 6], PDE inhibitors [7–13], or modulation of adenylyl cyclase through activation or blockade of specific membrane receptors [14, 15] have been shown to modulate cell proliferation. Imidazo[1,2-*a*]pyrazine derivatives display PDE inhibitory properties [16–19]. Some of these compounds have already been shown to exhibit antiproliferative properties on human lymphocytes [7] and human erythroleukemic cancer cells [20] and to display a cytotoxic action on the Dami cell line [21].

The aim of the present work was to study the effect of a series of non-cytotoxic imidazo[1,2-*a*]pyrazine derivatives on the growth of the Dami cell line [22], which has been shown to be negatively regulated by cAMP [6]. Their action on cell growth was studied in relation with their effect on intracellular cAMP content, their inhibitory potency on PDE isoenzymes isolated from Dami cells, their physicochemical properties, and in particular their purine-like structure and lipophilicity.

MATERIALS AND METHODS

Materials

Dami cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Horse serum was purchased from Techgen International (Les Ulis, France), and cell culture medium and reagents were from Gibco BRL (Eragny, France). Pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid tetrasodium (PPADS) was obtained from RBI (Bioblock, Illkirch, France). The cAMP ^{125}I-radioimmunoassay kit was from Immunotech (Marseille, France), and the CytoTox 96 kit was from Promega

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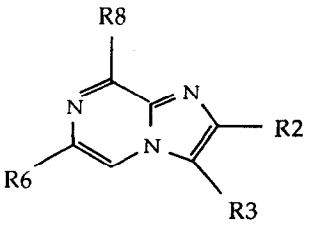
‖ *Abbreviations:* IBMX, isobutylmethylxanthine; LDH, lactate dehydrogenase; PDE, phosphodiesterase; PGE1, prostaglandin E1; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid tetrasodium.

Received 17 July 1996; accepted 23 December 1996.



	R2	R3	R6	R8
PAB12	H	H	Br	NH_2
PAB30	H	CH_2OH	Br	$NHCH_3$
PAB40	H	CH_2OCH_3	Br	$NHCH_3$
SCA40	CN	H	Br	$NHCH_3$
SCA41	CN	H	Br	NHC_2H_5
SCA44	CN	H	Br	NH c-pentyl

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

(Charbonnières, France). Radiochemicals were obtained from Amersham (Les Ulis, France). The imidazo[1,2-*a*]pyrazine derivatives (Fig. 1) were synthesized in our laboratory as previously described [16]. Other reagents were purchased from Sigma (St. Quentin Fallavier, France).

Cells and Cell Culture Conditions

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% nonessential amino acids. They were incubated in disposable sterile erlenmeyer flasks (Corning, Polylabo, Strasbourg, France) at 37° with a humidified atmosphere of 5% CO_2. Cultures were fed every 2–3 days by partial replacement of spent medium.

Proliferation Studies

Cellular proliferation was studied by placing cells in the exponential phase of growth in microwells (Nunc, Polylabo, Strasbourg, France) 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 counting performed using a Coulter Counter ZM (Coultronics S. A., Margency, France) equipped with a 140-μm orifice tube and calibrated with 14-μm latex particles.

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 [23]. Cells were seeded and incubated with the different compounds for 48 h as described by Vittet *et al.* [6].

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° before addition of compounds to be tested. Incubations were performed at 30° 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 × *g* for 2 min, and the supernatants were neutralized 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 2,500 × *g* for 5 min. The neutralized extracts were stored at −20°. cAMP concentration was assayed according to the manufacturers' recommendations.

PDE Assay

PDE isoenzymes were separated from Dami cell cytosol by anion exchange chromatography on DEAE-Sepharose CL-6B. Three types of 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 types III, IV, and V, respectively. The PDE activity was determined by the method of Thompson *et al.* [24] modified by Cook *et al.* [25]. Assays were performed at 37° 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 guanosine 3′,5′-cyclic monophosphate (cGMP), pH 8.0), and 25 μL compound to be tested or their respective solvent. Following 30 min of incubation, the reaction was stopped by transfer 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° 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 1 × 8–400 resin and incubation on ice for 30 min. After centrifugation (2500 × *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 any assay. The concentration of the PDE inhibitors that causes 50% PDE inhibition (IC_{50}) was determined from concentration-response curves. At least two concentration-response curves were generated for each agent.

Statistical Evaluation

Results are means ± SEM. Statistical differences were appreciated by Student's *t* test or Whitney-U test (cAMP assay), $p < 0.05$ being considered as significant. IC_{50}

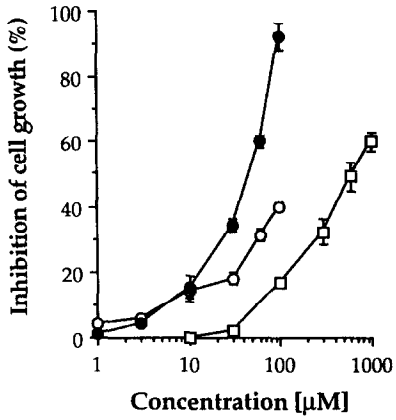


FIG. 2. Concentration-dependent activity of imidazo[1,2-*a*]pyrazine derivatives SCA41 (○), SCA44 (●) and IBMX (□) on Dami cell growth. Each point represents the mean ± SEM of three experiments performed in duplicate. Each value of duplicate is based on four cell counts.

values were calculated using computer software [26] and lipophilicity values logP were evaluated by the method of Viwanadhan *et al.* [27].

RESULTS

Effects of Imidazo[1,2-a]pyrazine Derivatives and Isobutylmethylxanthine (IBMX) on Dami Cell Proliferation

SCA41, SCA44, and IBMX induced concentration-dependent cell growth inhibition (Fig. 2, Table 1). Because of the poor compound solubility, the highest concentrations tested (100 μM and 1000 μM) could not be exceeded. SCA44 was ten times more potent on cell growth inhibition than IBMX. PAB40 and SCA40 only induced a weak (20.4% ± 1.9 and 24.7% ± 1.8, respectively) growth inhibition at highest concentration tested, while PAB12 and PAB30 had no activity (less than 9% of control at the highest concentration). None of the compounds assayed induced cytotoxicity, as seen by the lack of increased LDH release (results not shown).

Effects of Imidazo[1,2-a]pyrazine Derivatives and IBMX on Intracellular cAMP Levels in Presence and Absence of Prostaglandin E1 (PGE1)

In an attempt to a potential relationship between cell growth inhibition and intracellular cAMP concentrations, we investigated the effect of the compounds on cAMP concentration in presence and absence of the adenylyl cyclase stimulator PGE1. PGE1 was tested at 0.1 μM, a concentration that induced only a small cAMP increase in Dami cells (37.7 ± 11.4 pmol/10^6 cells). In the absence of PGE1, all derivatives except PAB12 significantly increased cAMP concentration (Fig. 3A). Among them, SCA40, SCA41 (100 μM), and IBMX (1 mM) induced the most potent increase. SCA40, SCA41, SCA44, and IBMX increased cAMP levels in a concentration-dependent manner (Fig. 4). All compounds significantly potentiated PGE1-induced cAMP formation, the most potent being SCA41 (Fig. 3B).

Effects of Imidazo[1,2-a]pyrazine Derivatives and IBMX on Dami Cell Proliferation in Presence and Absence of Prostaglandin E1 (PGE1)

Activity of the imidazo[1,2-*a*]pyrazine derivatives on cell proliferation in the presence of PGE1 is shown in comparison with their own effects in Table 2. 0.1 μM PGE1 elicited weak proper antiproliferative effects (11.6% ± 0.5). Imidazo[1,2-*a*]pyrazine derivatives and PGE1 displayed additive effects (SCA44 and SCA41) or small potentiating action (PAB40 and SCA40).

Effects of Imidazo[1,2-a]pyrazine Derivatives and IBMX on PDE Isoenzymes Types III, IV, and V Isolated from Dami Cells

All compounds exhibited PDE-inhibitory effects on the three PDE isoenzymes isolated from Dami cells. IC_{50} values toward PDE types III, IV, and V indicated different types of inhibitors among the imidazo[1,2-α]pyrazine derivatives (Table 1) tested (1. non-selective: PAB12, SCA41, and SCA44; 2. preferences for type III: SCA40 or type IV:

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

	−log (IC_{50})*			
Compounds†	G.I.	Type III	Type IV	Type V
PAB12	<4.0	<4.0	<4.0	<4.0
PAB30	<4.0	4.0 ± 0.1	5.6 ± 0.1	<4.0
PAB40	<4.0	5.0 ± 0.1	6.5 ± 0.2	<4.0
SCA40	<4.0	6.4 ± 0.2	5.3 ± 0.1	5.1 ± 0.1
SCA41	<4.0	5.9 ± 0.1	5.3 ± 0.1	5.4 ± 0.1
SCA44	4.5 ± 0.1	4.8 ± 0.1	5.3 ± 0.1	5.1 ± 0.1
IBMX	3.2 ± 0.1	4.8 ± 0.1	4.6 ± 0.1	4.6 ± 0.1

* Values were the means ± SEM of 6–13 experiments performed in triplicate.

† Compounds were assayed with 1 μM cAMP (types III and IV) or cGMP (type V) as substrate on isoenzymes isolated from Dami cells.

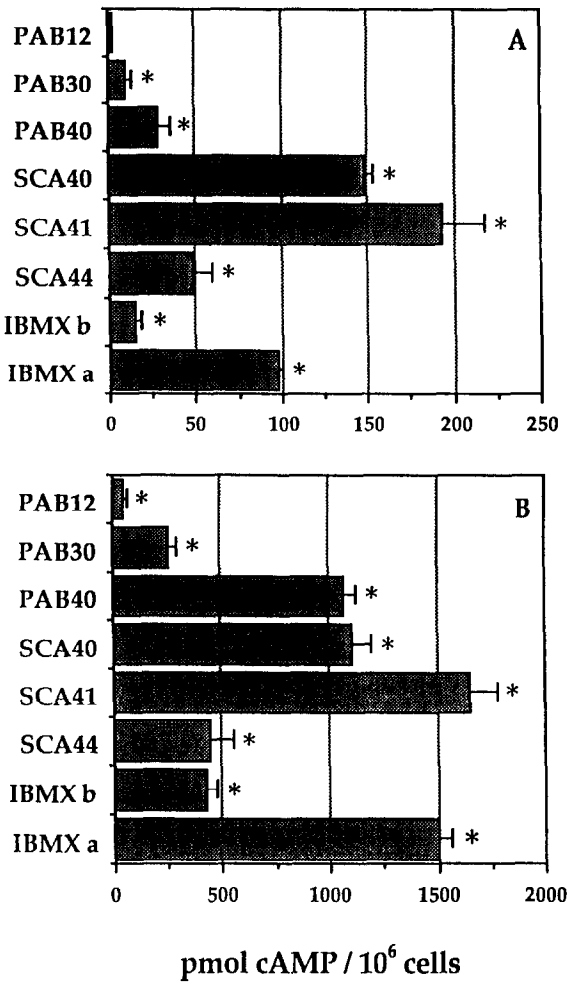


FIG. 3. Effects of imidazo[1,2-*a*]pyrazine derivatives (100 μM) and IBMX (a, 1000 μM and b, 100 μM) on intracellular cAMP levels (in absence (A) and in presence (B) of 0.1 μM PGE1). Histograms are the means ± SEM of three to five experiments performed in duplicate and represent the differences between cells treated with the test compounds and their respective control, which depends on the solvent used (values in pmol/10^6 cells): (A) 26.6 ± 0.9 (PAB12, PAB30), 37.4 ± 3.1 (IBMX), 33.5 ± 1.2 (PAB40, SCA40, SCA41, SCA44); (B) 68.2 ± 10.0 (PAB12, PAB30), 61.7 ± 6.4 (IBMX), 147.8 ± 26.3 (PAB40, SCA40, SCA41, SCA44). *$p < 0.01$.

PAB30 and PAB40). IC_{50} values observed for the compounds with PDE type III or IV preferences were one to two logs higher than those of IBMX. PAB12 elicited only weak effects on the three isoenzymes with IC_{50} levels higher than 100 μM.

Effects of Imidazo[1,2-a]pyrazine Derivatives on P_2 Purinoceptors

While the P_2 purinoceptor antagonist PPADS [28] fully antagonised ADP-induced growth inhibition (42.4% ± 0.9 at 500 μM), the antiproliferative action of the imidazo[1,2-*a*]pyrazine derivatives remained unchanged in the presence of this antagonist (Table 3).

Implication of the Physicochemical Properties of the Imidazo[1,2-a]pyrazine Derivatives

To study the implication of the lipophilicity of the imidazo[1,2-*a*]pyrazine derivatives in their antiproliferative action, that parameter was evaluated calculating their logP values [23]. There was a linear correlation ($R = 0.84$) between logP and the effects of the compounds (assayed at highest concentration) on cell proliferation (Fig. 5).

DISCUSSION

Among the six imidazo[1,2-*a*]pyrazine derivatives assayed on Dami cell proliferation, two inhibited cell growth in a concentration-dependent manner (SCA41 and SCA44), two (SCA40 and PAB40) were moderately effective, whereas the remaining two (PAB12 and PAB30) were practically devoid of effect. Very high concentrations up to 100 μM of imidazo[1,2-*a*]pyrazine derivatives were needed to induce Dami cell growth inhibition. These concentrations did not induce any toxic effect. Since some of the compounds studied here have been shown to display cAMP-elevating actions and PDE-inhibitory activity [16–19] and because PDE inhibitors inhibit proliferation of many cell types [7–13], we investigated whether imidazo[1,2-*a*]pyrazine derivatives were able to induce modifications in cAMP concentration without or with PGE1-induced adenylyl cyclase stimulation. Our purpose was indeed to know whether antiproliferative effects of the compounds could be related to their effects on cAMP or PDE. All products except PAB12 significantly increased cAMP levels at the highest concentration tested. As shown for SCA40, SCA41, SCA44, and IBMX, modulation of cAMP increase was concentration dependent and varied in the same direction as cell growth inhibition. However, it appeared that there was no correlation between the cAMP levels and the intensity of cell proliferation inhibition induced by the treatment. Indeed, SCA40 and SCA41 induced a very potent increase in cAMP levels without comparable effect on cell growth. In contrast, SCA44 and IBMX exhibited very potent effects on cell growth without a marked effect on cAMP levels. In addition, although the effects of SCA41 and SCA44 on intracellular cAMP levels were potentiated by PGE1, their antiproliferative activity was only adjoined by that of PGE1. Such a result again shows a lack of correlation between cell growth inhibition and cAMP levels. To verify a potential relationship between antiproliferative activity and selective PDE inhibitory potency, we have investigated the effect of the compounds on PDE isoenzymes type III, IV, and V isolated from Dami cells. PAB12, SCA41, and SCA44 elicited nonselective inhibitory effects on these isoenzymes. The other three derivatives (PAB30, PAB40, and SCA40) exhibited preferences for PDE type III (SCA40) or type IV (PAB30 and PAB40) isoenzymes, which was consistent with a previous study showing a selectivity on guinea pig atria and bovine trachea-isolated isoenzymes [18, 19]. There was a clear

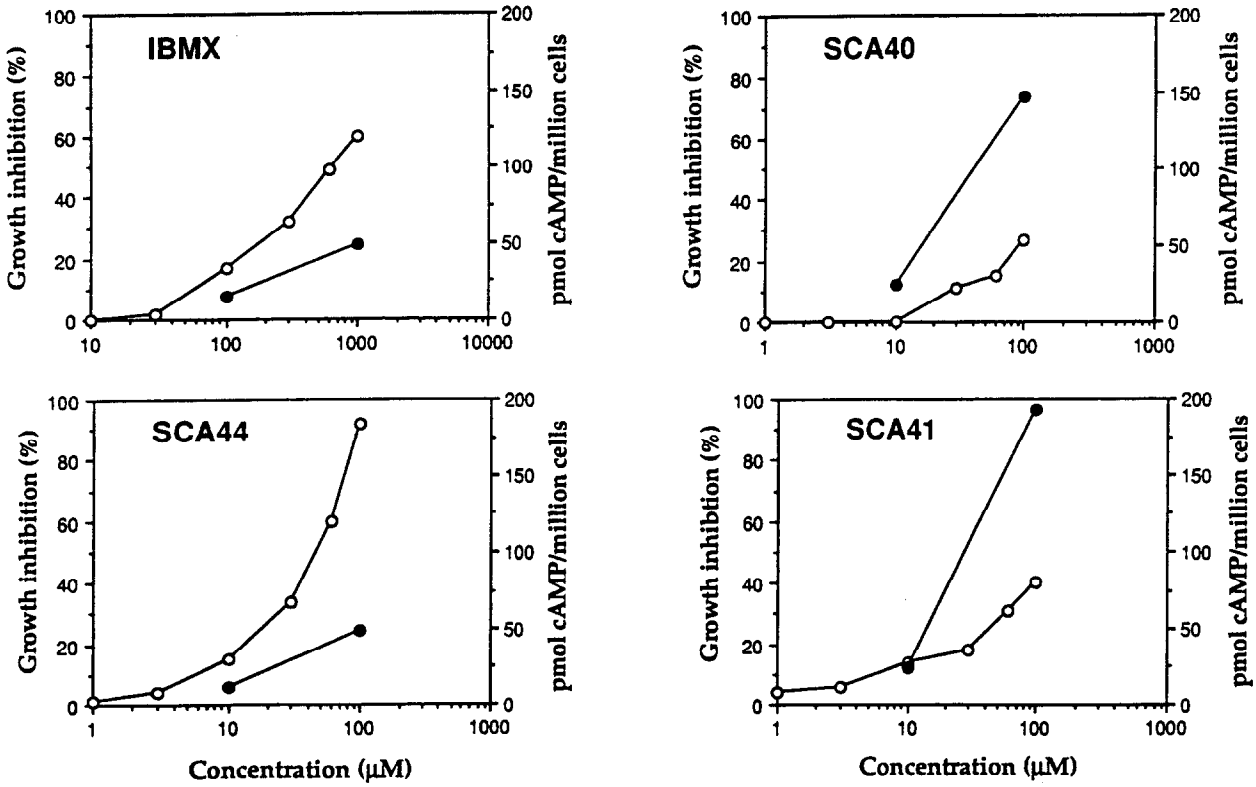


FIG. 4. Concentration-dependent variation of the two parameters: intracellular cAMP levels (●) and growth inhibition (○) for three selected imidazo[1,2-*a*]pyrazine derivatives and IBMX.

dissociation between the effects of the derivatives on cell proliferation and on PDE III and IV inhibition. In addition, a relationship between cGMP-PDE inhibition and cell proliferation appears unlikely in view of the equipotent effects of SCA40, SCA41, and SCA44 on PDE type V. This could be confirmed by the observation that two analogs of cGMP, 8-bromoguanosine 3',5'-cyclic monophosphate and 8-(4-chlorophenylthio)guanosine-3',5'-cyclic monophosphate Na-salt (both assayed at 1 mM), were ineffective on Dami cell proliferation (data not shown), whereas cAMP analogs elicited growth inhibitory effects [6]. It therefore appears that the effects of the compounds on Dami PDE isoenzyme activity and on intracellular cAMP levels could not explain their proliferation responses. These results are in contrast with other studies realised with nonselective and selective PDE inhibitors. The antiproliferative effect of the type IV inhibitor DC-TA-46 has been correlated with intracellular cAMP levels and PDE-inhibitory potency [8]. Moreover, for the type III inhibitor SK&F 94836 and type IV inhibitor Rolipram, alone or in combination, as well as for Cilostazol and Zardaverine, increased cAMP levels seemed to mediate inhibitory effects on cell growth [10–12]. Discrepancies between our results and those found previously suggest the implication of other parameters in addition to PDE inhibi-

TABLE 2. Effects of imidazo[1,2-*a*]pyrazine derivatives on Dami cell growth

Compounds*	G.I. (%)† in the absence of PGE1	G.I. (%) in the presence of PGE1 (0.1 μM)
PGE1	—	11.6 ± 0.5
PAB40	21.8 ± 2.1	40.4 ± 1.6‡
SCA40	24.9 ± 2.0	42.8 ± 2.8‡
SCA41	39.5 ± 2.6	57.2 ± 2.4
SCA41§	13.2 ± 2.2	32.0 ± 2.3
SCA44§	17.9 ± 2.9	31.2 ± 3.2

* 100 μM.
† G.I., growth inhibition; values are the means ± SEM; $n = 8$.
‡ Significant positive interaction; $p < 0.05$ (Two-way ANOVA).
§ 10 μM.

TABLE 3. Effects of imidazo[1,2-*a*]pyrazine derivatives on Dami cell growth

Compounds	G.I. (%)* in the absence of PPADS	G.I. (%)* in the presence of PPADS (100 μM)
PPADS	—	1.2 ± 0.5
ADP†	42.4 ± 0.9‡	1.8 ± 0.9
PAB40§	19.8 ± 2.3‡	19.1 ± 1.0‡
SCA40§	24.5 ± 3.2‡	26.1 ± 3.0‡
SCA41§	41.2 ± 2.9‡	40.7 ± 2.3‡
SCA44§	89.3 ± 1.7‡	87.0 ± 2.3‡

* G.I., growth inhibition; values are the means ± SEM of four to six individual experiments.
† 500 μM.
‡ Significantly different from control $p < 0.01$ (Student's t test).
§ 100 μM.

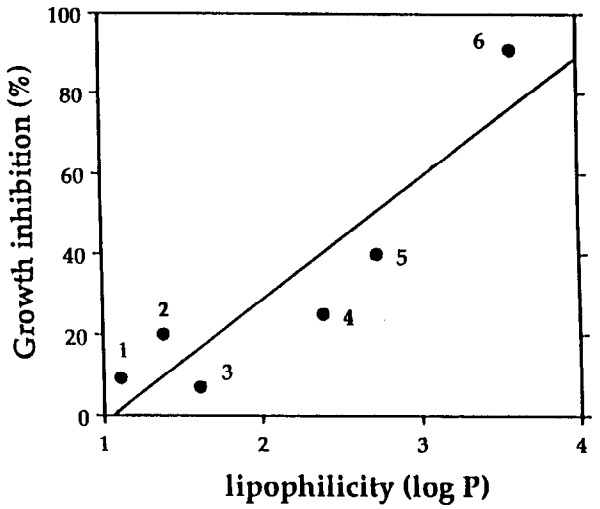


FIG. 5. Correlation (R = 0.84) between lipophilicity of imidazo[1,2-*a*]pyrazine derivatives and their activity on cell growth at the highest concentration assayed (100 μM). 1, PAB30; 2, PAB40; 3, PAB12; 4, SCA40; 5, SCA41; and 6, SCA44.

tion for the imidazo[1,2-*a*]pyrazine derivatives, including their chemical and physical properties.

Among other potential targets for imidazo[1,2-α]pyrazine derivatives are extracellular targets such as membrane receptors and in particular purinoceptors, in view of the chemical structure of the compounds. An action on adenosine receptors could not be evidenced [17, 29]. Considering ATP receptors, the presence of P_{2T} subtype on Dami cells has been recently described [30]. Furthermore, we have demonstrated herein an effect of ADP on Dami cell growth, antagonised by PPADS, a P_2-purinoceptor antagonist [28]. However, PPADS failed to modify the negative action of imidazo[1,2-*a*]pyrazine derivatives on cell growth, thus suggesting that P_2-purinoceptor activation was not implicated in their action.

Variation of the chemical structure appeared to be implicated in the modulation of antiproliferative action of the derivatives. Indeed, while the R3 substitutions seemed of minor importance, those of R2 and R8 greatly participated in the observed effect: the most active compounds have a carbonitril substitution in position 2. On the other hand, the length of the aliphatic substitutions in the 8-amino group led to both increased lipophilicity and antiproliferative action. The conjunction of both 2-carbonitril substitution and a long aliphatic chain in N8 made SCA44 the most active compound of the series. The link between lipophilicity and antiproliferative effect is suggested by the correlation between these parameters. This assertion is supported by the observation that in a series of cytotoxic imidazo[1,2-*a*]pyrazine derivatives, the length of the 8-aminoalkyl chain was also important in the cytotoxic action [21]. These results could explain why there was an important difference between IC_{50} values for compound effects on PDEs and on cell proliferation. Indeed, to reach their intracellular targets, including PDEs, the derivatives had to cross the plasma membrane, and it is likely that their concentration in the culture medium as well as that used in acellular assays was quite different from that obtained intracellularly. On the other hand, lipophilic compounds offer the advantage of reaching intracellular targets and therefore the physical nature of the products, driving their cell disposition, appeared to be the limiting step in their cellular effects, including action on cell growth. This could explain the lack of correlation between their effects on PDE and on growth inhibition. Other intracellular targets of the derivatives could also be implicated in the antiproliferative actions of PDE inhibitors, such as a direct action on cAMP-dependent protein kinase [31, 10] or on Gi protein [32]. Furthermore, there is evidence for a "cross-talk" between PDEs and other signalling systems that can regulate PDE activity and/or expression [33]. Such interactions or other targets have been suggested to modulate the PDE inhibitory activity of different PDE inhibitors studied [34–36].

Our observations show that some imidazo[1,2-*a*]pyrazine derivatives display an inhibitory effect on the growth of the malignant Dami cell line. The mechanism(s) by which this effect occurs appear(s) to involve several factors. The main and limiting factor seems, however, to be the variation in their lipophilicity.

These results demonstrate the pharmacological interest of these chemical structures and in particular that of SCA44 in the search for new anticancer drugs. Derivatives that possess a 2-carbonitril substitution associated with a longer aliphatic 8-amino substitution will be synthesised to increase antiproliferative effects.

The authors address special thanks to Dr. J. Bompart for calculation of logP values. The authors are also grateful to Dr. S.M. Greenberg for providing Dami cells.

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