New 5H-Pyridazino[4,5-b]indole Derivatives. Synthesis and Studies as Inhibitors of Blood Platelet Aggregation and Inotropics

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Some fused 5H-pyridazino[4,5-b]indoles (7–10), substituted in positions 1 and 4 by hydrazine and/or amino groups, have been synthesized. These new compounds present a planar topography, a dipole with an adjacent acidic proton, and a basic hydrogen-acceptor site opposite the dipole. These compounds have some resemblance to carbazeram and other pyridazino agents with cardiotonic activity. Some of the new compounds here described possess inotropic activity (Table I and II), with a complementary effect as inhibitors of platelet aggregation (Tables III and IV). 1-Hydrazino-4-(3,5-dimethyl)-1-pyrazolyl-5H-pyridazino[4,5-b]indole hydrochloride (7a·HCl) is the first compound described in the literature with activities as inhibitor of PDE-IV and as selective inhibitor of TXA_2 synthetase (Table V).

Recently several new positive inotropic agents are being developed in the clinical treatment of congestive heart failure.[1] Some of these, including carbazeram,[2] amrinone,[3] milrinone,[4] piroximone,[5] imazodan,[6] and sulmazole (See Chart I) are selective inhibitors of the adenosine 3',5'-cyclic phosphate phosphodiesterase (cAMP-PDE-IV), present in cardiac muscle. This inhibition is generally believed to be the principal mechanistic component of positive inotropic action for this cardiotonic family.

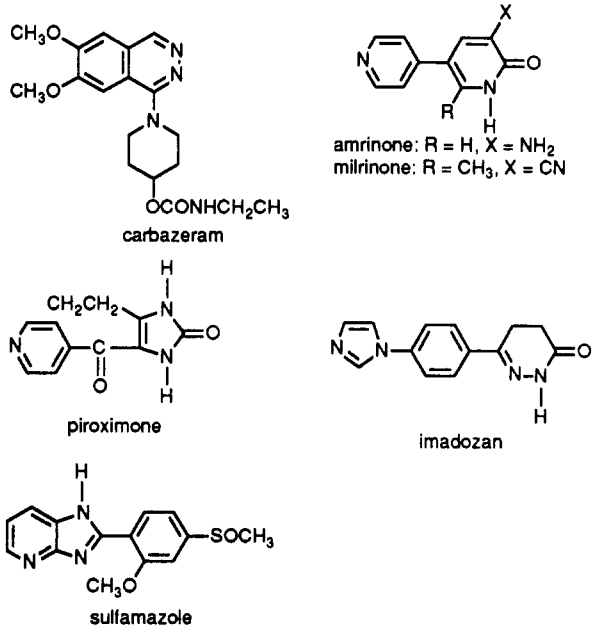
A second generation of cardiotonics are emerging with compounds that possess a good balance of inotropic and vasodilator activity, as well as additional actions that will retard or even reverse the progression of the disease and prolong the life of congestive heart failure patients. From that therapeutic perspective, the use of a cardiotonic agent with platelet aggregation inhibitory activity in patients with a myocardial infarction history and risk of coronary or pulmonary thrombosis[8,9] is advantageous.

On the basis of the fact that the PDE-IV platelet isoenzyme and the heart isoenzyme are identical,[10] the possibility of searching for products with inotropic and inhibitory activity of platelet aggregation was then considered.

The action of antithrombotic drugs that act on platelet aggregation has been largely due to the inhibition of platelet cyclooxygenase, the foremost example being acetylsalicylic acid (ASA).[11] A more effective approach is the selective inhibition of thromboxane A_2 synthetase (TXA_2).[12] TXA_2 is a potent vasoconstrictor and platelet-aggregating agent, which, under physiological conditions, rapidly hydrolyzes to TXB_2.[13] An additional advantage would be that some precursor of TXA_2, like prostaglandin H_2 (PGH_2), might increase the production of the vasodilator prostacyclin (PGI_2) in the vessel wall.[14] The control of the PGI_2/TXA_2 system may have a great biological significance in the treatment or prophylaxis of several cardiovascular diseases.[15] This control is particularly interesting for elderly people.[16] On the basis of this last consideration, our target will be centered on positive inotropic and vascular agents with platelet aggregation inhibitory activity as regulated by the PGI_2/TXA_2 system.

Recently, the synthesis and biological activity of a new series of pyridazino[4,5-b]indole derivatives have been reported.[17] 8-Methoxy-4-hydrazino-5H-pyridazino[4,5-b]indole is a new antihypertensive agent that inhibits the blood platelet aggregation through inhibition of the synthesis of TXB_2 and through stimulation of the synthesis of PGE_2 from arachidonic acid (AA). In this paper we

Chart I



† Medicinal Chemistry.
‡ Biochemistry.

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Table I. Structural[a] and Biological Properties

compd no.	R_1	R_4	PDE-IV % inhibn at 100 μM	IC_{50}, μM
4a	imidazolyl	imidazolyl	nd[c]	
4b	4-(2-methoxyphenyl)piperazinyl	4-(2-methoxyphenyl)piperazinyl	7.52 ± 1.42	
6	Cl	3,5-dimethylpyrazolyl	nd[c]	
7a	$NHNH_2$	3,5-dimethylpyrazolyl	9.39 ● 0.75	
7a·HCl	$NHNH_2$·HCl	3,5-dimethylpyrazolyl	–	348
7b	$NHNHCH_3$	3,5-dimethylpyrazolyl	44.75 ± 1.77	118
7c	morpholino	3,5-dimethylpyrazolyl	37.97 ± 0.97	
7d	1-piperidinyl	3,5-dimethylpyrazolyl	32.81 ● 2.12	
7e	4-(4′-methoxyphenyl)piperazinyl	3,5-dimethylpyrazolyl	4.80 ± 1.88	
7f	4-methylpiperazinyl	3,5-dimethylpyrazolyl	33.37 ● 2.68	
7g	4-(2′-ethoxyphenyl)piperazinyl	3,5-dimethylpyrazolyl	17.63 ● 3.06[b]	
7i	4-(2′-methoxyphenyl)piperazinyl	3,5-dimethylpyrazolyl	11.89 ● 2.82[b]	
7j	4-(4′-fluorophenyl)piperazinyl	3,5-dimethylpyrazolyl	2.52 ● 4.64[b]	
8	3,5-dimethyl-1-pyrazolyl	3,5-dimethylpyrazolyl	29.48 ● 2.26	
hydralazine			0	
amrinone (Wincoram)			52	126

[a] See Scheme I for basic structures. [b] 50 μM. [c] nd = no data.

present our results on new compounds related to pyridazino[4,5-*b*]indole with inotropic properties by way of an

Scheme I

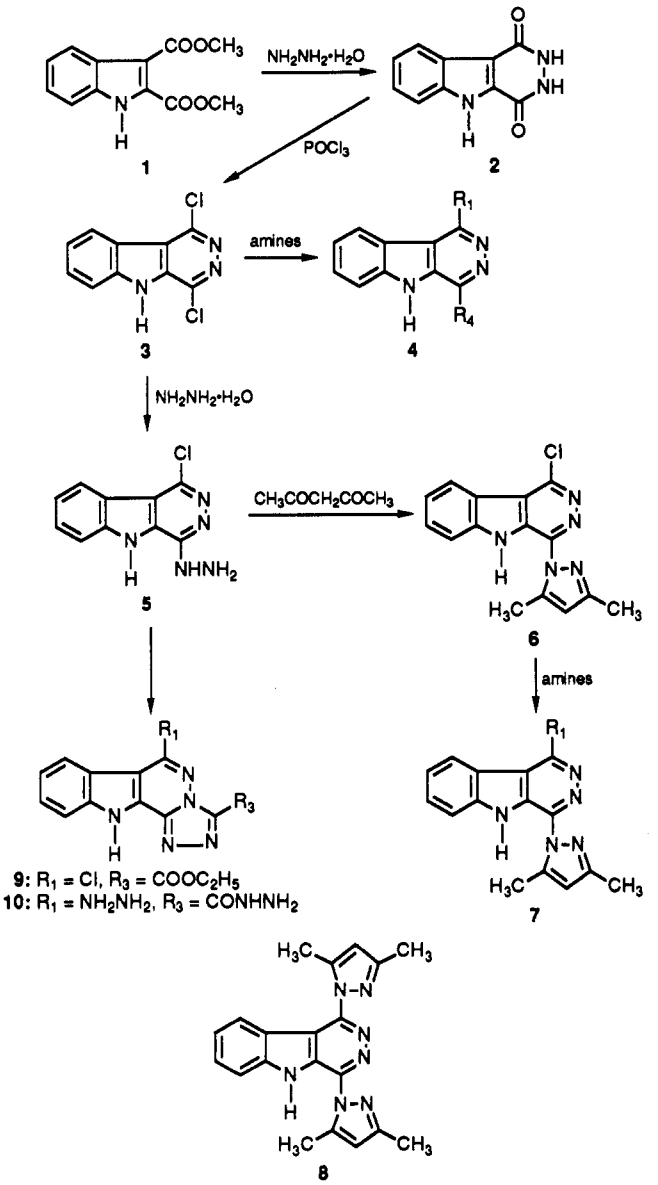

Table II.

compd	R_1	R_4	PDE-IV % inhibn at 100 μM	IC_{50}, μM
9	Cl	$COOC_2H_5$	36.95 ± 3.27	
10	$NHNH_2$	$CONHNH_2$	55.93 ± 3.19	121

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inhibitory mechanism of PDE-IV and antithrombotic properties with a decrease in the TXA_2 levels.

According to the model of PDE-IV inhibitors proposed by Moos et al.,[18] our compounds present a planar topography, a dipole with an adjacent acidic proton, and a basic hydrogen-acceptor site opposite the dipole, and also have some resemblance to carbazeran and other pyridazinone agents with cardiotonic activity. These essential requirements are valid for the 6-arylpyridazin-3(2H)ones activity as well as for their tricyclic analogues.[19]

The inclusion of an indole nucleus fused to a pyridazin nucleus is new in these activities. The hydrazino and, in general, the amino function were considered in order to achieve a peripheral α-blockade.

Chemistry

5*H*-Pyridazino[4,5-*b*]indole derivatives were prepared according to Scheme I. Methyl indole-2,3-dicarboxylate[20] (**1**) was reacted with 90% hydrazine hydrate to give a good yield of 1,2,3,4-tetrahydro-5*H*-pyridazino[4,5-*b*]indole-1,2-dione (**2**), which on heating with $POCl_3$ gave 1,4-dichloro-5*H*-pyridazino[4,5-*b*]indole (**3**).[21] This compound, on reaction with 90% hydrazine hydrate, gave 1-chloro-4-hydrazino-5*H*-pyridazino[4,5-*b*]indole (**5**) in good yield. **3** on reaction with different amines gave 1,4-diamino-5*H*-pyridazino[4,5-*b*]indoles (**4**). It was not possible to isolate monosubstitution products in this reaction.

With the aim of controlling the basicity of compound **5**, the hydrazine residue was incorporated into a pyrazole nucleus. 1-Chloro-4-(3,5-dimethyl-1-pyrazolyl)-5*H*-pyridazino[4,5-*b*]indole (**6**) was prepared quantitatively by reacting **5** with 2,4-hexanedione as reactant and solvent. When **6** was treated with different amines and hydrazine, the nucleophilic substitution compounds **7** were obtained.

Reaction of **5** with diethyl oxalate gave 3-(ethoxycarbonyl)-6-chloro-11*H*-1,2,4-triazolo[3,4-*c*]pyridazino-[4,5-*b*]indole (**9**), where the hydrazine residue was incorporated in a 1,2,4-triazine system. **9** on treatment with 90% hydrazine hydrate gave 3-carbazoyl-6-hydrazino-11*H*-1,2,4-triazolo[3,4-*c*]pyridazino[4,5-*b*]indole (**10**).

The introduction of the hydrazine residue in a pyrazole or a 1,2,4-triazole system has permitted the nucleophilic substitution of the additional chloro group. The physical properties of **4**, **7**, **9**, and **10** are presented in Tables I and II.

Biology

A. Inhibition of Phosphodiesterase, in Vitro Assay. In order to determine the inotropic activity of the new compounds, the study of their behavior on the enzymatic activity of phosphodiesterase-IV (PDE-IV) isolated from dog heart was measured. The separation of the different isoenzymes was carried out by using the Reeves and Cols method,[22] with a DEAE-Sepharose column. The PDE-IV inhibition study demonstrated that many compounds of this series show an outstanding degree of activity (Table I). **7b** (IC_{50} = 118 μM) and **10**·2HCl (IC_{50} = 121 μM) had IC_{50} values similar to that obtained for amrinone (Wincoram) (IC_{50} = 126 μM) in this assay. (Selection of amrinone as the reference compound was made because of its selectivity against the PDE-IV isoenzyme and *not* for structural reasons.)

Table III. Effect on Platelet Aggregation (Whole Blood)[a]

compd[b]	concn, M	% inhibn of platelet aggregation induced by[c] ADP[d]	AA[e]	AA
4a	5×10^{-4}	24.25 ± 5.40	98.88 ± 1.87	
	10^{-4}	23.25 ± 12.31	100	
4b	5×10^{-4}	64.00 ± 6.48	56.13 ± 8.47	
	10^{-4}	0	26.60 ± 2.50	
6	5×10^{-4}	31.00 ± 17.59	46.17 ± 8.71	
7a	5×10^{-4}	67.83 ± 22.30	92.14 ± 13.22	
	10^{-4}	nd	nd	
7a·HCl	5×10^{-4}	90.00 ± 18.40	97.00 ± 5.55	
	10^{-4}	23.00 ± 14.25	83.00 ± 32.19	
	5×10^{-5}	0	0	
7b	5×10^{-4}	33.80 ± 19.14	100	
	10^{-4}	0	7.86 ± 13.72	
7c	5×10^{-4}	47.83 ± 24.24	100	50
	10^{-4}	0	99.43 ± 3.11	
	5×10^{-5}	–	59.83 ± 14.84	
	10^{-5}	–	0	
7d	5×10^{-4}	64.83 ± 14.23	100	16.1
	10^{-4}	0	99.83 ± 0.43	
	5×10^{-5}	–	99.50 ± 1.29	
	10^{-5}	–	67.50 ± 10.37	
	5×10^{-6}	–	26.38 ± 13.03	
	10^{-6}	–	0	
7f	5×10^{-4}	89.25 ± 7.69	78.25 ± 4.16	
9	5×10^{-4}	57.63 ± 11.72	13.40 ± 11.53	
10·2HCl	10^{-4}	8.25 ± 23.40	38.33 ± 14.23	
ASA	5×10^{-3}	15.00 ± 12.75	30.00 ± 10.50	
	5×10^{-4}	100	100	
hydralazine[f]	5×10^{-4}	0	20.00 ± 12.00	
amrinone	5×10^{-4}	49.00 ± 8.59	100	

[a] See the Experimental Section for details. [b] Incubated for 60 min at 37 °C. [c] x ± SEM; $p \leq 0.05$ (n = 5–8). [d] 2.3×10^{-5} M. [e] 5×10^{-4} M.

B. Platelet Aggregation and Thromboxane Synthetase Inhibition Activities. The synthesized compounds were tested as potential inhibitors of platelet aggregation in guinea pig whole blood, with ADP and arachidonic acid (AA) as inducers of the aggregation. The compounds were tested at an initial concentration of 0.5 mM, with dimethyl sulfoxide (DMSO) as the solvent.

When the inhibition at this concentration was significant, concentrations were progressively decreased. The results have been summarized in Table III. The most active compounds were **7a**·HCl, **7c**, and **7d**. Determination of IC_{50} is made by way of the linear-regression method, starting from the data obtained with three concentrations. Generally, all the compounds of the **7** series that were tested showed significantly superior activity than that shown by ASA under our experimental conditions (equality of the test concentration).

The compounds that showed significant activity in whole blood were studied in human platelet-rich plasma (PRP)

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Table IV. Effect on Platelet Aggregation (PRP)[a]

compd	concn, M	% inhibn of platelet aggregation induced by ADP[c]	AA[d]	adrenaline[e]	collagen[f]
7a·HCl	$7 \times 5\ 10^{-5}$	23 ± 10.7	6.5 ± 4.0	22 ± 3.9	78.4 ± 13
	5×10^{-5}	22 ● 6.4	4.8 ± 2.6	0	0
7a	10^{-5}	nd[b]	nd	nd	nd
7c	10^{-5}	24.8 ● 12.9	3.35 ● 3.1	0	36.6 ● 2.7
7d	10^{-5}	23.9 ± 13.5	83.8 ± 12.6	42.5 ● 19.8	0
7b	5×10^{-5}	0	0	0	0
ASA	10^{-5}	20.0 ± 5.0	90.0 ± 2.0	38.3 ± 11.6	52.0 ± 24.5

[a] See the Experimental Section for details. [b] nd = no data. [c] 2×10^{-6} M. [d] 1.4×10^{-6} M. [e] 10^{-5} M. [f] 2 μg/mL.

Table V. Effect on Thromboxane Synthetase Activity on in Vitro Platelet Aggregation Induced by AA[a]

compd[b]	concn, M	% from basal values PGE_2	TXB_2
4a[c]	5×10^{-4}	119	−31
	10^{-4}	518	−61
7a[c]	5×10^{-4}	96	−25
7a·HCl	5×10^{-4}	11.80 ● 15.47	−91.2 ± 6.32
7b	5×10^{-4}	−72.80 ● 15.19	−93.00 ● 5.31
7c	5×10^{-4}	−78.00 ± 9.26	−95.67 ± 4.17
	10^{-4}	−80.00 ● 10.00	−95.00 ± 10.00
7d	5×10^{-4}	−80.40 ± 18.11	−95.20 ● 6.01
	10^{-4}	−91.00 ● 2.00	−100
	5×10^{-5}	−100	−100
ASA	5×10^{-3}	−100	−100

[a] See the Experimental Section for details. [b] n = 5–8. [c] n = 3.

in aggregation induced by ADP, AA, adrenaline, and collagen. The initial results obtained from a concentration of 10 μM (Table IV) indicate a greater activity for **7d**, especially when the aggregation was induced by AA.

From the samples in which an inhibition ≥85% in the whole blood platelet aggregation induced by AA was obtained, the potential selective inhibitory effect of the TXA_2 synthetase enzyme was studied. The prostaglandin E_2 (PGE_2) and thromboxane B_2 (TXB_2) levels were determined by RIA techniques. **7a**·HCl provoked an increase (12%) in the PGE_2 levels, and a decrease (91%) of TXB_2 (see Table V). Compounds **4a** and **7a** provoked a significant increase in the PGE_2 levels.

Conclusions

We have synthetized and tested "in vitro" some fused indole[4,5-*b*]pyridazines—substituted in positions 1 and 4 by hydrazine and other amino groups—as inhibitors of platelet aggregation and as inotropics. We have demonstrated that some members belonging to this series of compounds possess inotropic activity, with a complementary effect as inhibitors of the platelet aggregation.

Starting from our experimental data, one can deduce, at first glance, the following conclusions: (1) The tricyclic fused system of pyridazino[4,5-*b*]indole has some intrinsic cardiotonic and antiaggregatory activities. (2) Amino substituents placed in position 1 of the pyridazine ring act as inotropics (PDE-IV inhibition) in a way similar to that shown by amrinone (Wincoram), which is taken as reference. (3) 1-Hydrazino-4-(3,5-dimethyl-1-pyrazolyl)-5*H*-pyridazino[4,5-*b*]indole hydrochloride (**7a**·HCl) selectively inhibits the enzyme thromboxane A_2 synthetase. It should be taken into consideration that hydralazine, a structure which is closely related to those included in this study, has not shown (in our experimental model) antiaggregant activity against ADP and AA, nor has it demonstrated any activity as an inhibitor of PDE-IV at the concentrations tested. (4) To our knowledge **7a**·HCl is the first compound, described in the literature, that is a mixed PDE-IV/TXA_2 synthetase inhibitor which gives a better hemodinamic profile than a pure inotropic agent. Reduction of the respective IC_{50} in the diverse activities seems to be possible through molecular modification.

Experimental Section

All the new compounds were characterized by elemental analysis, IR spectra, and ^1H NMR spectra. The IR spectra for all compounds were recorded on a Perkin-Elmer 681 instrument, using KBr tablets. The ^1H NMR spectra were obtained on a Brücker AC-200E, a Perkin-Elmer R-32 (90-MHz), or a Perkin-Elmer R-24 (60-MHz) instrument with Me_4Si as the internal standard at a concentration of about 0.1 g/mL. The IR and ^1H NMR spectra were consistent with assigned structures. Melting points were determined on a hot plate of a microscope (Reisert's apparatus) and are uncorrected. Elemental analysis (Carlo Erba elemental analyzer) obtained from vacuum-dried samples (over P_2O_5 at 1–2 mmHg, 24 h at about 60–80 °C) were within 0.4% of the theoretical values.

Dimethyl Indole-2,3-dicarboxylate (1). This compound was obtained according to previously reported methods,[20] as white needles, with a yield of 75%: mp 114 °C (MeOH); NMR (DMSO-d_6, 60 MHz) δ 3.85 (6 H, d, CH_3), 7.20–7.60 (3H, m, H_5, H_6, H_7), 7.85–7.95 (1 H, m, H_4), 12.65 (1 H, bs, NH indole); IR 1730 (s, C=O), 1690 (s, C=O) cm^{-1}.

1,2,3,4-Tetrahydro-5*H*-pyridazino[4,5-*b*]indole-1,4-dione (2) was obtained from 1 according to previously reported methods,[21] as a white solid: 75% yield; NMR (DMSO-d_6, 90 MHz) δ 7.30–7.75 (3 H, m, H_6, H_7, H_8), 8.2–8.3 (1 H, d, H_9), 10.45 (2 H, bs, CONH), 12.64 (1 H, s, NH indole); IR 1650 (s, C=O), 1630 (m, C=N) cm^{-1}.

1,4-Dichloro-5*H*-pyridazino[4,5-*b*]indole (3). A mixture of **2** (2.0 g, 0.01 mol) and $POCl_3$ (40 mL) was heated at 90 °C for 4 h. The solid that precipitated upon cooling was filtered and washed with H_2O (5 × 50 mL), Et_2O (5 × 50 mL), and EtOH (5 × 50 mL), to give **3**[21] as white solid: 2.1 g, 90% yield; mp 250 °C dec; NMR (DMSO-d_6, 90 MHz) δ 7.4–7.85 (3 H, m, H_6, H_7, H_8), 8.37 (1 H, d, H_9), 13.60 (1 H, bs, NH indole); IR 3060–3180 (m), 1620 (m), 660 (m, CCl) cm^{-1}. Anal. ($C_{10}H_5Cl_2N_3$) H, N; C: calcd, 50.42; found, 49.94.

1,4-Diamino-5*H*-pyridazino[4,5-*b*]indoles (4). These compounds were obtained by boiling a mixture of **3** (1.0 g, 4.2 mmol), DMF (30 mL), and the corresponding amine (12 mmol) for 5–7 h. Upon cooling of the reaction mixture, the desired compound crystallized out. The yields were about 40–60%. The following compounds were obtained.

1,4-Diimidazolyl-5*H*-pyridazino[4,5-*b*]indole (4a): from 3 and imidazole; 0.58 g, 48% yield; white solid; mp >300 °C (DMF); NMR (DMSO-d_6, 200 MHz) δ 7.38–8.60 (m, 10 H, Ar + imidazole), 13.00 (s, 1 H, NH); IR 3100 (m, NH) 2700–3000, (bs, CH), 1620 (m, C=N) 1420 (s, CN), 7.30 (s, 1,2-disubst) cm^{-1}. Anal. ($C_{16}H_{11}N_7$) C, H, N.

1,4-Bis[4-(2-Methoxyphenyl)piperazinyl]-5*H*-pyridazino[4,5-*b*]indole (4b): from 3 and 1-(2-methoxyphenyl)piperazine; 1.38 g, 60% yield; cream solid; mp 159–160 °C (ETOH/H_2O); NMR (DMSO-d_6, 200 MHz) δ 3.3 (s, 8 H, piperazine), 3.5 (s, 8 H, piperazine), 3.8 (s, 6 H, CH_3), 6.7–6.1 (s, 8 H, Ar), 7.4 (t, 1 H, H_8), 7.5 (t, 1 H, H_7), 7.7 (d, 1 H, H_9), 8.04 (d, 1 H, H_6), 12.2 (s, 1 H, NH); IR 3020 (w, NH), 2980–2730 (bs, CH_2, CH_3), 1490 (s, CN), 1230 (s, CO), 730 (1, 1,2-disubst) cm^{-1}. Anal. ($C_{32}H_{35}N_7O_2$) C, H, N.

1-Chloro-4-hydrazino-5*H*-pyridazino[4,5-*b*]indole (5) was obtained from 3 according to methods previously reported:[20] 85%

yield; this compound was insoluble in organic solvents; mp >235 °C dec; NMR (DMSO-d_6, 90 MHz, 125 °C) δ 4.6–5.4 (2 H, bs, NH_2), 7.3–7.85 (4 H, m, H_6, H_7, H_8, H_9), 8.35 (1 H, d, NH); IR 1650 (m), 1620 (m), 740 (m) cm^{-1}.

1-Chloro-4-(3,5-dimethyl-1-pyrazolyl)-5*H*-pyridazino[4,5-*b*]indole (6). 1-Chloro-4-hydrazino-5*H*-pyridazino[4,5-*b*]indole (5; 2.4 g, 0.01 mol) was mixed with acetylacetone (25 mL) and refluxed for 8 h, when the product dissolved. The excess acetylacetone was then removed in vacuum, to give 6 as white solid: 3.2 g, 84% yield; mp 191 °C (2-PrOH); NMR (DMSO-d_6, 200 MHz) δ 2.4 (3 H, s, CH_3), 2.6 (3 H, s, CH_3), 6.2 (1 H, s, CH), 12.6 (1 H, s, NH indole); IR 3.300 (m), 2900 (m), 1620 (s, C=N) cm^{-1}. Anal. ($C_{15}H_{12}N_5Cl$) C, H; N: calcd, 23.53; found, 24.01.

1-Amino-4-(3,5-dimethyl-1-pyrazolyl)-5*H*-pyridazino[4,5-*b*]indoles (7). 1-Hydrazino-4-(3,5-dimethyl-1-pyrazolyl)-5*H*-pyridazino[4,5-*b*]indole (7a). A mixture of 100% hydrazine hydrate (10 mL) and 6 (3 g, 1 mmol) was refluxed for 6 h. The solid that precipitated upon cooling was filtered and recrystallized from 2-PrOH: 2.05 g, 70% yield; mp 177 °C (2-PrOH); NMR (DMSO-d_6, 200 MHz) δ 2.3 (3 H, s, CH_3), 2.4 (3 H, s, CH_3), 3.3 (2 H, bs, NH_2), 6.1 (1 H, s, CH), 7.2–8.4 (6 H, m, H_6, H_7, H_8, H_9, NH); IR 2900–3000 (m), 1620 (m, C=N), 3200–3400 (bs, NH) cm^{-1}. Anal. ($C_{15}H_{15}N_7$) C, H; N: calcd, 33.45; found, 33.03.

1-Hydrazino-4-(3,5-dimethyl-1-pyrazolyl)-5*H*-pyridazino[4,5-*b*]indole Hydrochloride (7a·HCl). A mixture of **7a** (2.8 g, 1 mmol) and 25% HCl (aqueous) (50 mL) was refluxed for 2 h. During the process, the compound dissolved and the hydrochloride precipitated upon cooling. The solid was collected by filtration, thereby obtaining a cream product, **7a**·HCl: 2.85 g, 90% yield; mp 269 °C (EtOH); IR 3300–3100 (bs, $NHNH_2$), 1590 (m, C=H), 750 (s, 1,2-disubst) cm^{-1}. Anal. ($C_{15}H_{16}N_7Cl·H_2O$) C, H, N.

1-(*N'*-Methylhydrazino)-4-(3,5-dimethyl-1-pyrazolyl)-5*H*-pyridazino[4,5-*b*]indole (7b). A mixture of 6 (3 g, 1 mmol) and methylhydrazine (5 mL) was refluxed for 6 h. It was then cooled and the white solid obtained was collected and recrystallized from EtOH: 2.08 g, 70% yield; mp 165 °C; NMR (DMSO-d_6, 200 MHz) δ 2.5 (3 H, s, CH_3), 2.7 (3 H, s, CH_3), 3.5 (3 H, d, CH_3), 6.4 (1 H, s, CH), 7.4–8.9 (7 H, m, H_6, H_7, H_8, H_9, NH); IR 3400 (m), 2850–3000 (m), 1620 (s, C=O) cm^{-1}. Anal. ($C_{16}H_7N_7$) C, H, N.

1-Morpholino-4-(3,5-dimethyl-1-pyrazolyl)-5*H*-pyridazino[4,5-*b*]indole (7c). 1-Chloro-4-(3,5-dimethyl-1-pyrazolyl)-5*H*-pyridazino[4,5-*b*]indole (6; 1.5 g, 5 mmol) was dissolved in hot 2-PrOH. Morpholine (5 mL) was added and the mixture refluxed for 6 h. The solvent was removed in vacuum, thereby **7c** was obtained as a white solid: 1.21 g, 70% yield; mp 215 °C (EtOH); NMR (DMSO-d_6, 90 MHz) δ 2.6 (3 H, s, CH_3), 2.7 (3 H, s, CH_3), 3.4–4 (8 H, m, CH_2 morpholine), 6.3 (1 H, s, CH), 7.4–8.1 (5 H, m, H_6, H_7, H_8, H_9, NH); IR 3200 (m, NH), 2980–2840 (m) 1620 (m, C=N), 740 (m, 1,2-disubst) cm^{-1}. Anal. ($C_{19}H_{20}N_6O$) C, H, N.

1-Piperidino-4-(3,5-dimethyl-1-pyrazolyl)-5*H*-pyridazino[4,5-*b*]indole (7d). 1-Chloro-4-(3,5-dimethyl-1-pyrazolyl)-5*H*-pyridazino[4,5-*b*]indole (6; 1.5 g, 5 mmol) was dissolved in hot 2-PrOH. Piperidine (5 mL) was added and the mixture refluxed for 6 h. The solvent was removed in vacuum. The beige solid obtained was recrystallized from EtOH: 1.18 g, 70% yield; mp 211 °C; NMR (DMSO-d_6, 90 MHz) δ 1.8 (4 H, bs, CH_2), 2.4 (3 H, s, CH_3), 2.6 (3 H, s, CH_3), 3.5 (6 H, bs, CH_2), 6.2 (1 H, s, CH), 7.3–8.1 (4 H, m, H_6, H_7, H_8, H_9), 13.2 (1 H, s, NH); IR 3400 (m, NH), 2850–3000 (m), 1620 (s, C=N), 740 (s, 1,2-disubst) cm^{-1}. Anal. ($C_{20}H_{22}N_6$) C, H; N: calcd, 24.28; found 24.70.

1-[4-(4-Methoxyphenyl)piperazinyl]-4-(3,5-dimethyl-1-pyrazolyl)-5*H*-pyridazino[4,5-*b*]indole (7e). A mixture of 6 (2.0 g, 6.7 mmol), 1-(4-methoxyphenyl)piperazinyl succinate, and sodium carbonate (4.0 g, 37.7 mmol) in DMF (40 mL) was refluxed for 20 h. The mixture was precipitated with H_2O under magnetic stirring, thereby a brown precipitate was obtained which was collected through filtration. It was then washed with abundant hot water. The purification and isolation of the desired product was achieved through a chromatographic column. Kieselgel 60 (Merck 7734) was used as the stationary phase and a mixture made up of petroleum ether and ethyl acetate as the mobile phase (9:1, for the extraction of 6, and 7:3, so as to speed up the outflow of the product formed). The fractions extracted from the column containing the new product were concentrated through rotatory evaporation. This gave **7e** as a white solid: 0.57 g, 20% yield; mp 145–149 °C (EtOH); NMR (DMSO-d_6, 200 MHz) δ 2.38 (3 H, s, CH_3), 2.61 (3 H, s, CH_3), 3.35 (4 H, s, CH_2), 3.64 (4 H, s, CH_2), 3.71 (3 H, s, CH_3O), 6.25 (1 H, s, CH), 6.87 (2 H, d, CH Ar), 7.04 (2 H, s, Ar), 7.49 (1 H, t, H_8), 7.64 (1 H, t, H_7), 7.93 (1 H, d, H_9), 8.06 (1 H, d, H_6), 11.91 (1 H, s, NH indole); IR 3378 (s, NH) 2924–2819 (bs, aliph), 1623 (m, C=N), 1418 (s, CN), 1230 (s, CO), 757 (m, 1,2-disubst) cm^{-1}. Anal. ($C_{26}H_{27}N_7O$) H, N; C: calcd, 68.87; found, 69.29.

1-(4-Methylpiperazinyl)-4-(3,5-dimethyl-1-pyrazolyl)-5*H*-pyridazino[4,5-*b*]indole (7f). Compound 6 (0.5 g, 1.6 mmol) was suspended in 1-methylpiperazine (10 mL) in the presence of Na_2CO_3 (0.5 g, 4.7 mmol). The mixture was then refluxed for 7 h, after which Na_2CO_3 was separated by filtration. The filtrate was then left to cool and the product was precipitated by the addition of water. The solid was then collected by filtration in vacuum and washed with abundant H_2O: 0.28 g, 49.5% yield; mp 131.5 °C (MeOH/H_2O), NMR (DMSO-d_6, 200 MHz) δ 2.40 (3 H, s, CH_3), 2.46 (3 H, s, CH_3), 2.80 (3 H, s, CH_3), 3.35 (4 H, s, CH_2), 3.67 (4 H, s, CH_2), 6.08 (1 H, s, CH), 7.40 (1 H, t, H_8), 7.5 (1 H, t, H_7), 7.6 (1 H, d, H_9), 8.02 (1 H, d, H_6), 10.92 (1 H, s, NH); IR 3320 (s, NH), 3000–2800 (bs, CH aliph), 1640 (m, C=N), 1430 (s, CN), 760 (s, 1,4-disubst) cm^{-1}. Anal. ($C_{20}H_{23}N_7$) C, H, N.

1-[4-(2-Methoxyphenyl)piperazinyl]-4-(3,5-dimethyl-1-pyrazolyl)-5*H*-pyridazino[4,5-*b*]indole (7i). A mixture of 6 (2 g, 6.7 mmol), 1-(2-methoxyphenyl)piperazine monohydrochloride (4.7 g, 20.5 mmol), sodium carbonate (2.5 g, 23.6 mmol), and DMF (20 mL) was refluxed over 25 h and then left to cool. The mixture was precipitated with H_2O under magnetic stirring, giving a brown precipitate that was collected and washed with hot H_2O. The desired product was isolated by chromatography [Kieselgel 60, Merck 7734, as the stationary phase and a mixture (8:2–8:3) of petroleum ether and AcOEt as the mobile phase]: 3.49 g, 11.5% yield; mp 139–143 °C (EtOH); NMR (DMSO-d_6, 200 MHz) δ 2.39 (3 H, s, CH_3), 2.63 (3 H, s, CH_3), 3.38 (4 H, s, 2 CH_2), 3.67 (4 H, s, 2 CH_2), 3.83 (1 H, s, OCH_3), 6.25 (1 H, s, CH), 7.48 (1 H, t, H_8), 7.64 (1 H, t, H_7), 7.94 (1 H, d, H_9), 7.98 (4 H, m, CH, Ar), 8.09 (1 H, d, H_6), 11.92 (1 H, s, NH indole); IR 3416 (s, NH), 2900–2831 (bs, aliph), 1619 (m, C=N), 1422 (s, CN), 1238 (s, CO), 747 (s, 1,2-disubst) cm^{-1}. Anal. ($C_{26}H_{27}N_7O$) C, H, N.

1-[4-(2-Ethoxyphenyl)piperazinyl]-4-(3,5-dimethyl-1-pyrazolyl)-5*H*-pyridazino[5,4-*b*]indole (7g). A mixture of 6 (2 g, 6.7 mmol), 1-(2-ethoxyphenyl)piperazine (4.89 g, 20.2 mmol), sodium carbonate (2.5 g, 23.5 mmol), and DMF (40 mL) was refluxed over 11 h, following which, the mixture was precipitated with H_2O under magnetic stirring. The brown precipitate that appeared was collected and washed with abundant H_2O. The isolation and purification of the desired product was achieved through chromatography (stationary phase, Kieselgel 60; mobile phase, petroleum ether/AcOEt 9:1–8:2–7:3): 0.46 g, 14.3% yield; mp > 150 °C (EtOH/H_2O, sandy powder); NMR (DMSO-d_6, 200 MHz) δ 1.35 (3 H, t, CH_3), 2.35 (3 H, s, CH_3), 2.59 (3 H, s, CH_3), 3.32 (4 H, s, CH_2), 3.62 (4 H, s, CH_2), 4.03 (2 H, c, CH_2), 6.23 (1 H, s, CH), 6.90 (4 H, s, Ar), 7.43 (1 H, t, H_8), 7.61 (1 H, t, H_7) 7.90 (1 H, d, H_9), 8.06 (1 H, d H_6), 11.88 (1 H, s, NH indole); IR 3366 (s, NH), 2824 (bs, CH aliph), 1620 (m, C=N), 1426 (s, CN), 1239 (s, CO), 742 (m, 1,2-disubst) cm^{-1}. Anal. ($C_{27}H_{29}N_7O$) C, H, N.

1-[4-(4-Fluorophenyl)piperazinyl]-4-(3,5-dimethyl-1-pyrazolyl)-5*H*-pyridazino[4,5-*b*]indole (7j). 1-Chloro-4-(3,5-dimethyl-1-pyrazolyl)-5*H*-pyridazino[4,5-*b*]indole (2.0 g, 6.7 mmol) was made to react with 1-(4-fluorophenyl)piperazine dihydrochloride (3.4 g, 13.4 mmol) in presence of sodium carbonate (284 g, 30.2 mmol) in DMF (20 mL). The mixture was refluxed during some 40 h. The product was precipitated with H_2O and then collected by filtration in vacuum and washed with abundant H_2O: 1.98 g, 67.6% yield; light yellow needles; mp 184.4–185 °C (EtOH); NMR (DMSO-d_6, 200 MHz) δ 2.45 (3 H, s, CH_3), 2.68 (3 H, s, CH_3), 3.51 (4 H, s, 2CH_2), 3.70 (4 H, s, 2 CH_2), 6.31 (1 H, s, CH), 7.17 (4 H, m, Ar), 7.52 (1 H, t, H_8), 7.70 (1 H, t, H_7), 8.0 (1 H, d, H_9), 8.13 (1 H, d, H_6), 12.00 (1 H, s, NH indole); IR, 3173 (m, NH), 1446 (s, CN), 851 (m, p-subst), 780 (s, 1,2-disubst) cm^{-1}. Anal. ($C_{25}H_{24}N_7F$) C, H, N.

1,4-Bis(3,5-dimethyl-1-pyrazolyl)-5*H*-pyridazino[4,5-*b*]-indole (8). A mixture of **7a** (3.0 g, 10 mmol) and acetylacetone (10 mL) was refluxed over 8 h with magnetic stirring. Following

this, it was left to cool and the excess acetylacetone was removed through rotatory evaporation under reduced pressure. The solid obtained was washed with H_2O (10 × 25 mL) and recrystallized from 2-PrOH/H_2O: 2.32 g, 65% yield; mp 157–158 °C; NMR (DMSO-d_6, 200 MHz) δ 2.36 (6 H, s, CH_3), 2.43 (3 H, s, CH_3), 2.73 (3 H, s, CH_3), 6.31 (1 H, s, CH), 6.34 (1 H, s, CH), 7.30 (1 H, t, H_8), 7.7 (2 H, m, H_7, H_9), 7.98 (1 H, d, H_6), 12.24 (1 H, s, NH indole); IR 3305 (s, NH), 2964–2963 (m, CH), 1623 (m, C=N), 7.39 (s, 1,2-disubst) cm^{-1}. Anal. ($C_{20}H_{19}N_7$) C, H, N.

3-(Ethoxycarbonyl)-6-chloro-11*H*-1,2,4-triazolo[3,4-*c*]-pyridazino[4,5-*b*]indole (9). A suspension of 5 (4.67 g, 0.02 mmol) in EtOH (50 mL) was boiled while being stirring. Then, diethyl oxalate (10 mL, 0.07 mol) was added in portions. The mixture was refluxed for 8 h and then cooled at room temperature. The solid that precipitated was collected and recrystallized: 5.35 g, 85% yield; mp >300 °C (EtOH); yellow solid; NMR (DMSO-d_6, 90 MHz) δ 1.4 (3 H, t, CH_3), 4.5 (2 H, c, CH_2), 7.3–8.5 (4 H, m, Ar), 11.8 (1 H, s, NH); IR 3200–3050 (bs), 1740 (s), 1620 (m), 1280 (s), 750 (s) cm^{-1}. Anal. ($C_{14}H_9N_5O_2$·HCl) C, H, N.

3-Carbazoyl-6-hydrazino-11*H*-1,2,4-triazolo[3,4-*c*]-pyridazino[4,5-*b*]indole (10). A mixture of 9 (3.15 g, 0.01 mol), EtOH (50 mL), and hydrazine hydrate (5 mL) was refluxed for 8 h and then left to cool. The solid was collected and washed with hot EtOH and DMF. This gave the required compound in a yield of 2.37 g (80%): mp >300 °C (EtOH/DMF); white solid); NMR (TFA, 90 MHz) δ 7.5–8.6 (m, 4 H, H_6, H_7, H_8, H_9); IR 3400–2800 (bs), 1670 (s), 1620 (s), 740 (s) cm^{-1}. Anal. ($C_{12}H_{11}N_9O$) C, H, N; C: calcd, 48.48; found, 47.81.

Isolation of Phosphodiesterases (PDE) and Assay of Activity. Four peaks of cyclic nucleotide phosphodiesterase activity (DPE I, PDE II, PDE IV, and PDE V)[23,24] from dog heart were separated by chromatography on DEAE-Sepharose-Cl-6B (Pharmacia Fine Chemicals) by using a procedure essentially similar to the one described by Reeves et al.[22] for the separation of PDE activity from human and guinea pig cardiac ventricle. Dog heart tissue (25 g) was homogenized in 250 mL of 20 mM Bis-Tris, 5 mM 2-mercaptoethanol, 2 mM benzamidine, 2 mM EDTA, and 50 mM sodium acetate, pH 6.5, with a Polytron instrument (three times at setting 16.5 for 10 s). Phenylmethanesulfonyl fluoride was dissolved in propan-2-ol and added to the buffer immediately before homogenization, giving a final concentration of 50 μM. The homogenate was centrifuged for 30 min at 105000*g* and the supernatant applied to a column (11 × 2.5 cm) of DEAE-Sepharose-CL-6B, preequilibrated with homogenization buffer. The column was washed with 100 mL of homogenization buffer and the PDE activities were eluted with a linear gradient of 0.05–1.0 M sodium acetate in the homogenizing buffer. A flow rate of 80 mL/h was used throughout the ion-exchange chromatography; 10-mL fractions were collected and assayed for PDE activity. Four activities could be distinguished on the basis of elution profiles measured at 0.5 μM cyclic AMP, 25 μM cyclic AMP, 0.5 μM cyclic AMP + 0.1 mM amrinone, and 0.5 μM cyclic AMP + 2 μM cyclic GMP.

Peaks III and IV (PDE-IV and V) were separated once more (by chromatography) in order to improve their purification. The peaks were collected, diluted with an equal volume of homogenization buffer without sodium acetate, applied to a column (17 cm × 1.5 cm) preequilibrated with the homogenization buffer with 350 mM sodium acetate (instead of 50 mM), and eluted with a linear gradient of 350 mM–1 M sodium acetate in the same homogenization buffer.

Peak I (PDE-I) is a high V_{max} PDE activity which corresponds to the Ca^{2+}/calmodulin-stimulated PDE; peak II (PDE-II) is a cGMP-stimulated cAMP-PDE; peak III (PDE-IV) and peak IV (PDE-V) are both high-affinity cAMP-PDE. The former (subtype IV) is a cGMP-inhibitable form which is also sensitive to the cardiotonic PDE inhibitors, whereas the later (subtype V) is a cGMP-noninhibitable form which is quite insensitive to the cardiotonic agents but strongly inhibited by rolipram.[24]

PDE activity was assayed by the batch method of Thompson et al.[25] All the chemicals were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the reaction medium was 2.5%. IC_{50} values were obtained for peak III (PDE-IV) by incubation of the enzyme at 1 μM c-AMP and by a range of inhibiting concentrations from 1.0 μM to 0.1 mM.

Platelet Aggregation Using Guinea Pig Whole Blood. Guinea pig whole blood was obtained by direct cardiac puncture of anesthetized (Et_2O) female guinea pigs weighing ≥450 g. Blood was collected in 4.5-mL portions in Vacutainer 64625 silicone-coated tubes containing 0.5 mL of 3.8% sodium citrate. Normally blood from five to eight guinea pigs was collected.

Aggregation tests were run with the chronolog whole blood aggregometer, following the Cardinal and Flowers method.[26] The operating principle of the whole blood aggregometer involves measurement of the electrical impedance between two electrodes immersed in the whole blood sample. When platelets aggregate in the whole blood aggregometer, they coat the electrodes to a greater or lesser degree, thereby impeding the current between the electrodes to an extent proportional to the amount of aggregation that has occurred 5 min after the addition of the aggregating agent (ADP, 27 μM; AA, 0.5 mM) with stirring. Test drugs were dissolved in DMSO (6.6 μL final volume DMSO/mL in cuvette). Citrated whole blood was distributed in 500-μL aliquots into aggregometer cuvettes containing 500 μL of normal saline. Test drug solutions or DMSO were added (6.6 μL) and the cuvettes were incubated for 60 min at 37 °C. Incubation was followed by the addition of aggregating agents (50 μL). The extent of aggregation of test samples was compared to the extent of aggregation of control samples and is expressed as "percent of control".

Effects on Thromboxane Synthetase Activity on in Vitro Platelet Aggregation. The demonstration of selective inhibition effects of the compounds was determined according to the modified Gorman model.[27] PGE_2 and TXB_2 levels in the test samples, following aggregation, were determined by radioimmunoassay (RIA) according to methods previously reported.[28] RIA studies were performed on the whole blood samples that experienced inhibited platelet aggregation, following compound inhibition and addition of AA.

In Vitro Platelet Aggregation Using Human Platelet-Rich Plasma. Blood was collected from volunteers who had not ingested drugs within the preceding 2 weeks and had not eaten 9 h before the blood draw.

Blood was collected in 4.5-mL portions in Venoject silicone-coated tubes containing sodium citrate (1 part of citrate to 9 parts of blood). Platelet-rich plasma (PRP) was prepared by centrifuging the citrated blood at 880 rpm for 10 min at room temperature and then separating. The remaining blood was centrifuged at 2500 rpm over 20 min at room temperature to obtain the platelet-poor plasma (PPP). The PRP was adjusted with PPP to a count of 300 000 platelets/mL. The platelet content of the PRP was determined with a coulter-thrombocounter.

Platelet aggregation was measured by the absorbance method of Born and Cross[29] with a platelet aggregation meter. Test drugs were dissolved in normal saline and 10% ethylene glycol (EG). Other lower concentrations were prepared by serial dilution in saline. PRP, adjusted to 300 000 platelets/mL, was distributed in 200-μL aliquots into silicone-coated cuvettes (8 × 50 mm).

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Addition of a drug solution or saline/10% EG (25 μL) was followed with incubation over 60 min at 37 °C. Then the aggregating agent (25 μL; 2 μM ADP, 0.1 μM adrenaline, 2 μg/mL collagen, 1.4 μM AA) was added and the aggregating effect carried out over 5 min with stirring at a controlled temperature of 37 °C. Appropriate concentrations of aggregating agents were determined by an initial titration. Each compound was studied with several normal plasmas.

The extent of aggregation of test samples was compared with the extent of aggregation of control samples and is expressed as "percent of control".

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Peptide Derivatives Specific for a *Plasmodium falciparum* Proteinase Inhibit the Human Erythrocyte Invasion by Merozoites

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A specific proteinase of *P. falciparum* merozoites has been detected by using hydrosoluble fluorogenic peptidic substrates synthesized by classical peptide chemistry; their N-terminal end was acylated by a gluconoyl group that protects them from aminopeptidase degradation and increases their hydrosolubility, and their carboxylic end was substituted by a 3-amino-9-ethylcarbazole group. The sequence Val-Leu-Gly-Lys was found to be the most specific substrate. On this basis, reversible peptidic inhibitors were synthesized by substituting the C-terminal lysyl residue, at the proteolytic site, by different alkylamines and amino alcohols. The activity of these compounds, studied on the *P. falciparum* proteinase and in in vitro cultures, strongly suggests a specific effect of this peptidic sequence on the reinvasion process. The peptidic inhibitors do not impair the release of merozoites from schizonts, but selectively inhibit the invasion step leading to the formation of rings. Although the natural target of this enzyme is not yet known, these specific peptide inhibitors could lead to a new antimalarial approach.

Introduction

The dramatic worldwide resurgence of malaria recently observed is mainly due to the spreading of antimalarial drug-resistant parasite strains and it is now clear that new therapeutic approaches are required. Researches to develop antimalarial vaccines are steaming ahead but several years will probably pass before this strategy will be an effective protection of human beings.

As the invasion of red blood cells by merozoites is a key event during malarial infection, the inhibition of this step appears as an attractive biological approach. In order to understand the molecular aspect of the highly efficient invasiveness of merozoites into erythrocytes, we were looking for specific parasite proteinases using various fluorogenic peptidic substrates. Proteinases have indeed been implicated in different steps of the *Plasmodia* life cycle and particularly in the release of merozoites and the invasion of erythrocytes (see Schrével et al.[1], for a review).

A neutral 68 kDa proteinase has been isolated in *Plasmodium berghei* (Pb 68)[2] and in *P. falciparum* (Pf 68)[3] schizonts. Its localization in the apex of *P. berghei* merozoites,[4] as well as its presence in free *P. falciparum* merozoites, arised the question of the biological role of this proteinase. As parasite proteinases could act on host erythrocyte proteins, the role of the Pf 68 proteinase during the reinvasion process of erythrocytes by merozoites was investigated.

. In the present paper, the synthesis of substrates specific for this *P. falciparum* proteinase is described: their N-terminal end was acylated by a hydrosolubilizing gluconoyl group[5] and their C-terminal end, at the proteolytic cleavage site, was substituted by a 3-amino-9-ethylcarbazole group (AEC). This fluorescent amine has been selected among several fluorescent reporter groups such as 4-methoxy-2-naphthylamine,[6] 7-amino-4-methylcoumarin,[7] or 7-amino-4-(trifluoromethyl)coumarin[8] for its interesting and particular spectroscopic properties,[9] its high sensitivity, and its very good coupling yield with amino acids. Furthermore, this amine allows quantitative determination of proteinases in cell lysates and/or supernatants.[10]

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