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# Progress in 5*H*[1]benzopyrano[4,3-d]pyrimidin-5-amine series: 2-methoxy derivatives effective as antiplatelet agents with analgesic activity

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**Abstract**

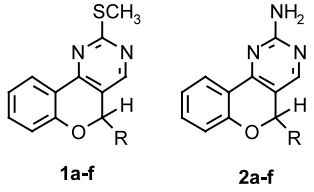
A series of 2-methoxy-5*H*[1]benzopyrano[4,3-d]pyrimidin-5-amines were prepared and screened for their in vitro antiplatelet activity inducing the aggregation by ADP, arachidonic acid (AA) and collagen. In vivo experiments were performed in order to evaluate their antiphlogistic, analgesic and antipyretic activities. Title compounds showed antiplatelet activity in aggregation AA or collagen-induced, and a good analgesic activity without any gastric toxicity. Comparison with a number of analogue benzopyrano[4,3-d]pyrimidine derivatives and some SAR consideration were reported. 

*Keywords:* 2-Methoxy-5*H*[1]benzopyrano[4,3-d]pyrimidin-5-amines; Antiplatelet activity; Analgesic activity

## 1. Introduction

Intravascular clot formation is an important factor in cardiovascular diseases such as myocardial infarction, unstable angina, deep vein thrombosis, the main cause of mortality in industrialised world; so, the prevention of hemostatic processes has become a major target for new therapeutic agents. Platelet aggregation is a fundamental step in the pathogenesis of thrombotic ischemic diseases and many clinical trials have evaluated the benefit of long-term use of antiplatelet drugs in reducing the incidence of these pathologies [1]. The cyclooxygenase inhibitor acetyl salicylic acid (ASA) has been the cornerstone for the secondary prevention of stroke, but presently other classes of antiplatelet drugs represent new opportunities to optimise the antithrombotic therapy [2,3]. The combination therapy with distinct antiplatelet agents targeting ADP receptors, platelet surface glycoproteins or platelet-dependent thrombin generation, such as ASA–dipyridamole, ASA–clopidrogel, or ASA–GPIIbGPIIIa inhibitors, has been tested in successful clinical trials [4]. Accordingly, the goal for current researches seems to be the development of new compounds interfering with different mechanisms in the platelet aggregation.

Recently, we reported the synthesis of a serie of 2-methylthio-5*H*[1]benzopyrano[4,3-d]pyrimidin-5-amines **1a**–**f** [5] containing some derivatives able to inhibit both arachidonic acid (AA) and ADP-stimulated aggregation of guinea-pig platelets in vitro unlike ASA. Moreover, a number of these compounds, are devoid of




| 1,2 | R |
|---|---|
| a | isopropylamino |
| b | t-butylamino |
| c | cyclopropylamino |
| d | pyrrolidino |
| e | piperidino |
| f | morpholino |

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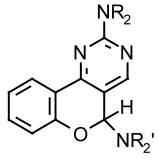
gastrolesivity resembling the gastrointestinal tolerability detected for 2-amino substituted analogues **2a**–**f** [6] previously published as effective NSAIDs with ASA-like antiplatelet profile.

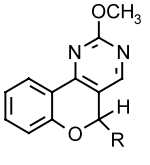
Considering these benzopyrano[4,3-d]pyrimidines useful leads for the development of new gastric-sparing antithrombotic drugs with combined anti-ADP–$TxA_2$ activity, we synthesised a series of 2-cycloamino-*5H*[1]benzopyrano[4,3-d]pyrimidin-5-amines **3a**–**i** [7].




**3a-i**

| 3 | $NR_2$ | $NR'_2$ |
|---|---|---|
| **a** | Pyrrolidino | Pyrrolidino |
| **b** | Piperidino | Pyrrolidino |
| **c** | Morpholino | Pyrrolidino |
| **d** | Pyrrolidino | Piperidino |
| **e** | Piperidino | Piperidino |
| **f** | Morpholino | Piperidino |
| **g** | Pyrrolidino | Morpholino |
| **h** | Piperidino | Morpholino |
| **i** | Morpholino | Morpholino |

Among these compounds those derivatives able to inhibit collagen-, ADP-, AA-induced platelet aggregation in vitro, evidenced an interesting antithrombotic activity in vivo, lacking hemorrhagic effects [8]. The pharmacological study so far performed with the various benzopyrano[4,3-d]pyrimidines strengthened the hypothesis that the presence of 2-methylthio substituent is important for antiplatelet activity against ADP which can be favourably affected by the concomitant 5-substitution with pyrrolidino moiety. With the aim of confirming the role of the 2-methylthio function in preventing the ADP-induced aggregation, we designed the 2-methoxy-*5H*[1]benzopyrano[4,3-d]pyrimidin-5-amines **4a**–**h**, isosteres of **1a**–**f**.




**4a-h**

| 4 | R |
|---|---|
| **a** | isopropylamino |
| **b** | t-butylamino |
| **c** | cyclopropylamino |
| **d** | diethylamino |
| **e** | pyrrolidino |
| **f** | piperidino |
| **g** | morpholino |
| **h** | benzylamino |

Their synthesis, in vitro antiplatelet activity and in vivo pharmacological screening for antiphlogistic/analgesic/antipyretic activity, along with some SAR considerations in comparison with previous compounds, are here reported.

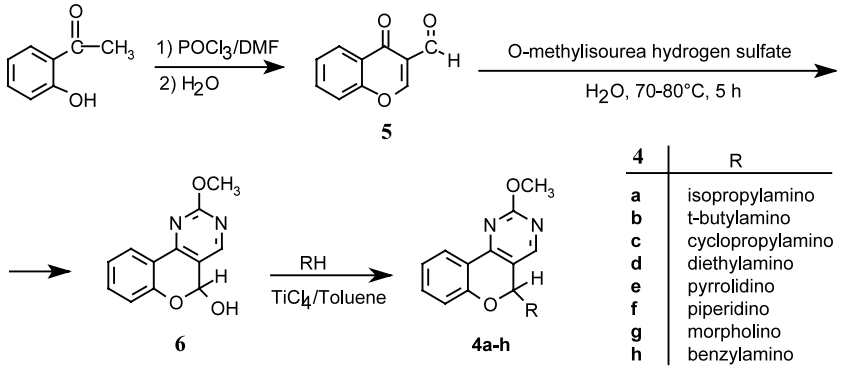
## 2. Chemistry

Compounds **4a**–**h** were prepared starting from 3-formylchromone **5**, obtained from 2-hydroxyacetophenone by a Vilsmeier reaction [9], which was condensed with *O*-methylisourea hydrogen sulfate to give the intermediate 2-methoxy-5-hydroxy-*5H*[1]benzopyrano[4,3-d]pyrimidine **6**. Then, the hemiacetalic hydroxy-group in position 5 of the benzopyrano[4,3-d]pyrimidine system was replaced with the suitable amines in the presence of $TiCl_4$, according to a method already described [5,6] to give the desired compounds **4a**–**h** (Scheme 1).

## 3. Pharmacology

All the new benzopyrano[4,3-d]pyrimidine derivatives were screened for their in vitro antiplatelet activity in guinea-pig platelet-rich plasma inducing the aggregation by ADP, AA and collagen. In vivo experiments were performed in order to evaluate their antiphlogistic, analgesic and antipyretic activities in rat paw oedema, mouse writhing test and rat *Escherichia coli*-induced pyrexia, respectively. Some selected compounds were tested also for the erosive effects on gastric mucosa in rats.

Compounds **4a**–**h** resulted devoid of antiplatelet activity in the ADP-induced aggregation while, on the contrary, they showed antiplatelet activity when the aggregation was induced by AA or collagen. The most active compounds **4f** (R = piperidino) and **4g** (R = morpholino) were as potent as ASA in inhibiting AA-induced aggregation.




**5**

**6**

**4a-h**

| 4 | R |
|---|---|
| **a** | isopropylamino |
| **b** | t-butylamino |
| **c** | cyclopropylamino |
| **d** | diethylamino |
| **e** | pyrrolidino |
| **f** | piperidino |
| **g** | morpholino |
| **h** | benzylamino |

Scheme 1.

Table 1
In vitro antiplatelet activity ($IC_{50}$ values in μM) of ASA and compounds **4a**–**h** on guinea-pig PRP platelet aggregation induced by ADP, AA and collagen

| Comp. | ADP (3 μM) induced aggregation | AA (50 μM) induced aggregation | Collagen (5 μg $ml^{-1}$) induced aggregation |
|---|---|---|---|
| ASA | nc | 61 ± 8 | 48 ± 9 |
| **4a** | (a) | 230 ± 25 | 82 ± 20 |
| **4b** | (a) | 382 ± 41 | 216 ± 44 |
| **4c** | nc | 235 ± 28 | 218 ± 62 |
| **4d** | nc | 215 ± 22 | 189 ± 52 |
| **4e** | (a) | 362 ± 35 | 370 ± 80 |
| **4f** | (a) | 68 ± 11 | 299 ± 67 |
| **4g** | (a) | 55 ± 7 | 120 ± 32 |
| **4h** | (a) | 448 ± 46 | 485 ± 75 |

(a) Inactive up to 1 mM; nc = not calculable because maximal inhibition of aggregation is lower than 50%.

Table 2
Comparison of analgesic potency expressed as $ID_{50}$ (the dose inhibiting the writhing response by 50% respect to the control group) and efficacy expressed as percentage of maximal inhibition of writhes induced by i.p. acetic acid in mice treated with compounds **4a**–**h** and **2a**–**f**

| Substituents in position 5 | Mouse writhing test | | | |
|---|---|---|---|---|
| | Comp. **4a**–**h** | | Comp. **2a**–**f** (a) | |
| | $ID_{50}$ (mg $kg^{-1}$ os) | Maximum inhibition (%) | $ID_{50}$ (mg $kg^{-1}$ os) | Maximum inhibition (%) |
| Isopropylamino | 186 | 55* | nc | 37* |
| *t*-Butylamino | 124 | 63* | 80 | 62** |
| Cyclopropylamino | 131 | 67* | 98 | 51** |
| Diethylamino | 21 | 85** | nt | nt |
| Pyrrolidino | nc | 7 | 61 | 72** |
| Piperidino | 132 | 67* | 95 | 52** |
| Morpholino | nc | 5 | 66 | 71** |
| Benzylamino | nc | 0 | nt | nt |

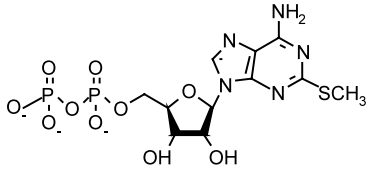
(a) Data from Bruno et al., Arzn. Forsch. Drug Res. 50 (2000) 140–147. Nt = not tested, nc = not calculable. *, $P < 0.05$; **, $P < 0.01$ significance as compared with controls.

With regard to the in vivo properties, some compounds **4** showed good analgesic activity, the most active being **4b** (R = *t*-butylamino), **4d** (R = diethylamino), **4c** (R = cyclopropylamino) and **4f** (R = piperidino), while they did not exhibit antipyretic activity. Finally, only compound **4c** (R = cyclopropylamino) showed a moderate antiphlogistic activity. The most active compounds did not affect gastric mucosa integrity after oral administration in rats.

The results of the pharmacological screenings are reported in Tables 1 and 2.

## 4. Results and discussion

Pharmacological screening of title compounds showed some interesting properties and allows a few remarks. Firstly, all 2-methoxy derivatives **4a**–**h**, differently from the 2-methylthio substituted isosteres **1a**–**f**, lacked antiplatelet activity when the aggregation was induced by ADP. Thus, we can conclude that the presence of a methylthio function in position 2 of the benzopyrano[4,3-d]pyrimidine system is very important to confer the antiplatelet activity when the ADP-dependent mechanisms are involved in the aggregation. A possible interaction of these 2-methylthio derivatives with ADP receptors could be speculated on the basis of the agonist activity toward ADP platelet receptor described for the structurally related compound 2-methylthio-ADP **7** [10].




**7**

Secondly, all the benzopyrano[4,3-d]pyrimidine derivatives inhibited platelet aggregation caused by both AA and collagen showing an ASA-like antiplatelet profile, although with a minor potency. In particular the most potent compound within this series was derivative **4g** confirming that the 5-substitution with the morpholino group supports the antiplatelet potency as already

observed for other benzopyrano[4,3-d]pyrimidine derivatives [7,8].

Moreover, compounds **4a**–**h** displayed a good analgesic activity while their antiphlogistic or antipyretic activities were moderate or absent. A remarkable anti-inflammatory activity was detected only for derivative **4c** which was less potent but as effective as indomethacin (10 mg $kg^{-1}$ os), in inhibiting rat paw swelling (about 40–45% inhibition;*, $P < 0.05$), while five compounds out of eight significantly prevented nociceptive response to intraperitoneal injection of acetic acid in mice, without displaying any gastrolesivity up to 200 mg $kg^{-1}$ os. By comparing the data concerning analgesic potency and efficacy of the different series of benzopyrano[4,3-d]pyrimidines so far studied it emerges that the insertion of the methoxy group in position 2 generates compounds **4a**–**h** still endowed with antinociceptive properties comparable to the corresponding 2-amino-derivatives **2a**–**f** mainly when in position 5 are present *t*-butylamino, cyclopropylamino or piperidino groups, but not when in the same position are present pyrrolidino or morpholino groups.

On the other hand, the 2-cycloamino substituted derivatives **3a**–**i** as well as the 2-methylthio substituted derivatives **1a**–**f** were fully inactive as analgesic agents [5–7]. In the present study, the highest analgesic potency was displayed by diethylamino derivative **4d** which almost completely suppressed writhing behaviour when administered at 5-fold higher dose than indomethacin ($ID_{50} = 21$ versus 3.6 mg $kg^{-1}$ for the reference drug).

In conclusion, these results attest that the substituent in position 2 plays a fundamental but not exclusive role in addressing the pharmacological activities towards antiplatelet or analgesic/antipyretic/antiphlogistic activities for the benzopyrano[4,3-d]pyrimidines. Indeed, it is noteworthy that also the nature of 5-substitution provides a further contribution to their potency and efficacy.

## 5. Experimental

### 5.1. Materials and chemical methods

All chemicals were obtained from Sigma-Aldrich S.r.L. (Milan, Italy).

Melting points are uncorrected and were measured with a Büchi 530 instrument (Büchi Laboratoriums-Technik AG, Flawil, Schweiz). IR spectra were recorded with a Perkin–Elmer 398 spectrophotometer (Perkin–Elmer, Milan, Italy). $^1$H NMR were recorded on a Varian Gemini 200 (200 MHz) instrument (Varian, Milan, Italy); chemical shifts are reported as $\delta$ (ppm) relative to tetramethylsilane (TMS) as internal stardard; the signals were characterised as s (singlet), d (doublet), t (triplet), m (multiplet), sept (septet); $J$ in Hz.

Reactions were followed by TLC on Kieselgel $60F_{254}$ (DC-Alufolien, E. Merck, Darmstadt, Germany). Analyses for C, H, N ($\pm$0.3% of the theoretical value), were determined with an Elemental Analyser EA 1110 (Fison-Instruments, Milan, Italy).

#### 5.1.1. Preparation of 5-hydroxy-2-methoxy-5H[1]benzopyrano[4,3-d]pyrimidine (**6**)

To a solution of *O*-methylisourea hydrogen sulfate (1.72 g, 10 mmol) in NaOH 2 N (10 ml), was added quickly 3-formylchromone (1.74 g, 10 mmol), $H_2O$ (20 ml), and $Et_3N$ (0.5 ml). The mixture was stirred for 5 h at 70–80 °C. The light red solid, obtained after cooling at room temperature (r.t.), was filtered and recrystallised from DMF–$H_2O$ (1:1). Yield: 1.80 g, 78%, m.p. 212–214 °C (lit. [11]: 219–220 °C).

IR($CHCl_3$): 3130 $cm^{-1}$ (OH). $^1$H NMR: $\delta$ 4.02 (s, 3H, $OCH_3$), 6.55 (d, $J = 4$, 1H, $H_5$, becames s after $D_2O$), 7.11–7.20 (m, 2H, $H_7 + H_8$), 7.53 (t, $J = 8$, 1H, $H_9$), 7.74 (d, $J = 4$, 1H, OH, disappears after $D_2O$), 8.22 (d, $J = 8$, 1H, $H_{10}$), 8.60 (s, 1H, $H_4$).

#### 5.1.2. General procedure for 5-alkyl–cycloalkyl–cycloamino-2-methoxy-5H[1]benzopyrano[4,3-d]pyrimidines (**4a**–**h**)

To a solution of anhydrous toluene (20 ml) and anisole (1 ml) cooled at 0 °C and stirred for 5 min, $TiCl_4$ (0.6 ml) was added quickly. Then, to the obtained red mixture a solution of the suitable amine (2.5 ml) in anhydrous toluene (2.5 ml), 5-hydroxy-2-methoxy-5*H*[1]benzopyrano-[4,3-d]pyrimidine **6** (1.5 g, 5 mmol) and a further solution of the same amine (1.5 ml) in anhydrous toluene (5 ml) were added in succession. The mixture was refluxed for 6 h. After cooling, conc. $NH_4OH$ (1.5 ml), 2-propanole (1 ml) and Kieselgur (1 g) were added in sequence, the suspension was stirred at r.t. for 10 min and filtered in vacuo. The solid was washed with warm toluene and $CH_2Cl_2$, the organic solutions were collected, washed with water, dried ($MgSO_4$) and concentrated under reduced pressure. The obtained raw product was purified by filtration on Florisil (100–200 mesh) ($CH_2Cl_2$ as eluent). The solvent was removed under reduced pressure and the obtained oil was induced to crystallise by adding ethyl ether. Finally, the crude solid was recrystallised from absolute ethanol.

**4a**: Yield 81%, white crystals, m.p. 96–97 °C. IR($CHCl_3$): 3325 $cm^{-1}$ (NH). $^1$H NMR: $\delta$ 1.09 (d, $J = 6$, 3H, $CH_3$), 1.17 (d, $J = 6$, 3H, $CH_3$) 2.12 (d, $J = 12$, 1H, NH, disappears with $D_2O$), 3.40 (sept, $J = 6$, 1H, CH), 4.07 (s, 3H, $OCH_3$), 6.03 (d, $J = 12$, 1H, $H_5$, becames s after $D_2O$), 6.98–7.13 (m, 2H, $H_7 + H_8$), 7.42 (t, $J = 8$, 1H, $H_9$), 8.25 (d, $J = 8$, 1H, $H_{10}$), 8.44 (s, 1H,

$H_4$). *Anal*. Calc. for $C_{15}H_{17}N_3O_2$: C, 66.40; H, 6.32; N, 15.49. Found: C, 66.60; H, 6.50; N, 15.45%.

**4b**: Yield 30%, white crystals, m.p. 112–113 °C. IR($CHCl_3$): 3245 $cm^{-1}$ (NH). $^1H$ NMR: $\delta$ 1.25 (s, 9H, $3CH_3$), 2.07 (d, $J=8$, 1H, NH, disappears with $D_2O$), 4.07 (s, 3H, $OCH_3$), 6.18 (d, $J=8$, 1H, $H_5$, becames s after $D_2O$), 6.96 (d, $J=8$, 1H, $H_7$), 7.10 (t, $J=8$, 1H, $H_8$), 7.42 (t, $J=8$, 1H, $H_9$), 8.27 (d, $J=8$, 1H, $H_{10}$), 8.40 (s,1H, $H_4$). *Anal*. Calc. for $C_{16}H_{19}N_3O_2 \cdot 0.75H_2O$: C, 64.30; H, 6.91; N, 14.06. Found: C, 64.31; H, 6.75; N, 13.78%.

**4c**: Yield 78%, white crystals, m.p. 78–79 °C. IR($CHCl_3$): 3350 $cm^{-1}$ (NH). $^1H$ NMR: $\delta$ 0.41–0.53 (m, 4H, $2CH_2$), 2.53–2.63 (m, 1H, CH) 2.84 (d, $J=12$, 1H, NH, disappears with $D_2O$), 4.07 (s, 3H, $OCH_3$), 5.97 (d, $J=12$, 1H, $H_5$, becames s after $D_2O$), 7.02–7.13 (m, 2H, $H_7+H_8$), 7.44 (t, $J=8$, 1H, $H_9$), 8.25 (d, $J=8$, 1H, $H_{10}$), 8.35 (s, 1H, $H_4$). *Anal*. Calc. $C_{15}H_{15}N_3O_2$: C, 66.90; H, 5.61; N, 15.60. Found: C, 66.74; H, 5.57; N, 15.63%.

**4d**: Yield 71%, white crystals, m.p. 50–51 °C. $^1H$ NMR: $\delta$ 1.08 (t, $J=7$, 6H, $2CH_3$), 2.83 (q, $J=7$, 4H, $2CH_2$), 4.09 (s, 3H, $OCH_3$), 6.22 (s, 1H, $H_5$), 6.92 (d, $J=8$, 1H, $H_7$), 7.04 (t, $J=8$, 1H, $H_8$), 7.38 (t, $J=8$, 1H, $H_9$), 8.22 (d, $J=8$, 1H, $H_{10}$), 8.40 (s, 1H, $H_4$). *Anal*. Calc. for $C_{16}H_{19}N_3O_2$: C, 67.35; H, 6.71; N, 14.73. Found: C, 67.69; H, 6.65; N, 14.66%.

**4e**: Yield 65%, white crystals, m.p. 51–52 °C. $^1H$ NMR: $\delta$ 1.73–1.79 (m, 4H, $2CH_2$ Pyrr), 2.80–3.03 (m, 4H, $2CH_2N$ Pyrr), 4.08 (s, 3H, $OCH_3$), 6.20 (s, 1H, $H_5$), 6.92–7.08 (m, 2H, $H_7+H_8$), 7.38 (t, $J=8$, 1H, $H_9$), 8.22 (d, $J=8$, 1H, $H_{10}$), 8.43 (s, 1H, $H_4$). *Anal*. Calc. for $C_{16}H_{17}N_3O_2$: C, 67.83; H, 6.05; N, 14.83. Found: C, 67.51; H, 6.02; N, 15.01%.

**4f**: Yield 73%, white crystals, m.p. 85–86 °C. $^1H$ NMR: $\delta$ 1.48–1.52 (m, 6H, $3CH_2$ Pip), 2.57–2.72 and 2.78–2.92 (2m, 4H, $2CH_2N$ Pip), 4.09 (s, 3H, $OCH_3$), 5.99 (s, 1H, $H_5$), 6.92–7.02 (m, 2H, $H_7+H_8$), 7.38 (t, $J=8$,1H, $H_9$), 8.21 (d, $J=8$, 1H, $H_{10}$), 8.41 (s, 1H, $H_4$). *Anal*. Calc. for $C_{17}H_{19}N_3O_2$: C, 68.67; H, 6.44; N, 14.13. Found: C, 68.30; H, 6.50; N, 13.88%.

**4g**: Yield 78%, white crystals, m.p. 116–117 °C. $^1H$ NMR: $\delta$ 2.62–2.77 and 2.81–2.96 (2m, 4H, $2CH_2$ N Morph), 3.65 (t, $J=5$, 4H, $2CH_2O$ Morph), 4.09 (s, 3H, $OCH_3$), 5.99 (s, 1H, $H_5$), 6.94–7.05 (m, 2H, $H_7+H_8$), 7.41 (t, $J=8$, 1H, $H_9$), 8.22 (d, $J=8$, 1H, $H_{10}$), 8.44 (s, 1H, $H_4$). *Anal*. Calc. for $C_{16}H_{17}N_3O_3$: C, 64.20; H, 5.72; N, 14.04. Found: C, 64.41; H, 5.74; N, 14.23%.

**4h**: Yield 55%, light yellow crystals, m.p. 76–77 °C. IR ($CHCl_3$): 3320 $cm^{-1}$ (NH). $^1H$ NMR: $\delta$ 2.50–2.70 (m, 1H, NH, disappears with $D_2O$), 4.08 (s, 3H, $OCH_3$), 4.12 (d, $J=4$, 2H, $CH_2$, becames s after $D_2O$), 5.96–6.02 (d, $J=12$, 1H, $H_5$, becames s after $D_2O$), 7.04–7.18 (m, 2H, $H_7+H_8$), 7.29–7.49 (m, 6H, $H_9+5H$ Ar), 8.26 (d, $J=8$, 1H, $H_{10}$), 8.51 (s,1H, $H_4$). *Anal*. Calc. for $C_{19}H_{17}N_3O_2$: C, 71.46; H, 5.37; N, 13.16. Found: C, 71.27; H, 5.21; N, 13.06%.

### 5.2. Pharmacological methods

The experiments were performed using male guinea-pigs (350–450 g) (Morini, S. Polo-RE, Italy) for in vitro tests, Wistar female rats (250–300 g) and Swiss female mice (25–35 g) for in vivo tests applying experimental procedures supervised and approved by the Care and Use Committees (DL 116/92).

#### 5.2.1. In vitro antiplatelet activity

According to the methods described in a previous paper [7] guinea-pig blood was obtained by cardiac puncture after $CO_2$ euthanasia and it was collected in plastic tubes containing nine parts blood:one part sodium citrate (3.8% w/v;). After centrifugation for 10 min at $200 \times g$ to obtain platelet rich plasma (PRP), from the remaining blood, which was centrifuged for 10 min at $2000 \times g$, platelet poor plasma (PPP) was produced. Platelet aggregation was performed in an Aggrecorder PA 3220 aggregometer (A. Menarini, Firenze, Italy) following the Born's turbidimetric method [12]. Aggregation was recorded as percent change in light transmission: the baseline was set by using PRP and full transmission (100%) was set by using PPP. PRP (250 μl) was preincubated at 37 °C for 5 min with solvent (dimethyl sulfoxide, DMSO), the compounds under study or the reference drug ASA (from $5 \times 10^{-5}$ to $1 \times 10^{-3}$ M) before the addition of the platelet aggregatory agent. PRP aggregation was induced by 3 μM ADP (25 μl) (Sigma), AA (50 μM) (Menarini) or collagen (5 μg $ml^{-1}$) and using concentrations sufficient to achieve maximum aggregation. Tests were performed within 3 h to avoid platelet inactivation. The effects of test compounds and acetylsalicylic acid used as reference drug were determined as percent inhibition calculated from the total aggregation in 5 min. Control samples received the same volume addition of DMSO at the final concentration of 0.5%. This concentration of solvent did not interfere with platelet assay.

#### 5.2.2. In vivo experiments

The test compounds were suspended in 0.5% methylcellulose and were orally administered at 100 mg $kg^{-1}$ in 0.5 ml/100 g body weight to rats and at 0.15 ml/100 g body weight to mice 1 h before the application of phlogogen, algogen and pyretogen agent. Indomethacin 10 mg $kg^{-1}$ os was used as reference drug in all the experimental tests while control animals received an equivalent volume of vehicle alone. Antiphlogistic, analgesic, antipyretic and gastrolesive activities were evaluated following the experimental procedures already described [6]. Briefly, antiphlogistic activity was studied

in rat by inducing paw oedema through subplantar injection of carrageenan, analgesic activity was evaluated in mice by acetic acid writhing test and antipyretic activity was determined in rat with *E. coli* LPS induced fever. The pharmacological activities were expressed as the percentage of inhibition calculated from the difference in the response between the treated and the control group at the time the maximum noxious effect occurred.

For the evaluation of gastrolesive effects, 5 h after drug (200 mg $kg^{-1}$) or vehicle oral administration, the animals were sacrificed by $CO_2$, the stomachs were removed and microscopically inspected in order to evaluate the gastric mucosal damage. The number and the length of gastric lesions were measured for each stomach by means of an image analyser system (Leitz, ASM 68K).

### 5.2.3. Statistical analysis

The results were expressed as mean $\pm$ SEM and the means were compared using Student's *t*-test, *, *P* value $< 0.05$ or **, $P < 0.01$ being considered as statistically significant or highly significant, respectively.

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