2-Amino/Azido/Hydrazino-5-alkoxy-*5H*-[1]benzopyrano[4,3-d]pyrimidines: Synthesis and Pharmacological Evaluation

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Abstract: New series of 5-alkoxy-benzopyranopyrimidine derivatives were developed from the chemical modulation of the substituent in position 2 of the scaffold, with the aim to produce analgesic/antiphlogistic agents more potent than analogues previously reported. The 2-hydrazino derivatives exhibited a good analgesic activity in writhing test; the analgesic doses of the compounds did not affect mice spontaneous locomotor activity thus any confounding sedative effect could be excluded. These derivatives revealed an aspirin-like profile with a strong inhibition of AA-induced platelet aggregation, probably due to a strong, non selective, inhibition of cyclooxygenases. In spite of the inhibition of COX activity displayed *in vitro*, the compounds did not cause gastric damage in rats after acute oral administration. A different pharmacological profile was observed for the 2-azido derivatives, particularly *in vivo*.

Key Words: 2-Amino/Azido/Hydrazino-5-alkoxy-*5H*-[1]benzopyrano[4,3-d]pyrimidines, analgesic activity, antinociceptive activity, antiphlogistic activity, antiplatelet activity, COX_1/COX_2 inhibition.

1. INTRODUCTION

According to World Health Organization, 90% of diseases are associated with pain (cancer, AIDS, arthro-pathologies, neuropathies, diabetes, etc…). The pain is a complex process involving multiple interrelated neurotransmitter systems both in the spinal cord and at supraspinal sites [1]. Currently, the therapies for pain consist mainly in the use of NSAIDs (Non Steroidal Anti-Inflammatory Drugs), opiates and analgesic adjuvants, such as antidepressants or local anesthetics. NSAIDs, inhibiting the cyclooxygenases enzymes COX-1 and COX-2, block the production of arachidonic acid derivatives, prostaglandins, prostacyclin and thromboxane, responsible for the peripheral sensitization and hyperalgesia, but they have no effect on acute nociception. The most important side effects of non-steroidal anti-inflammatory drugs are gastrointestinal ulceration and perforation, bleeding and nephrotoxicity. These adverse effects were associated with the COX-1 inhibition, thus in the last ten years a number of new COX-2 selective inhibitors were developed [2,3]. However, the CLASS (Celecoxib Long-term Arthritis Safety Study) [4] and VIGOR (Vioxx Gastrointestinal Outcomes Research) [5] trials, conducted by the manufacturers of celecoxib and rofecoxib, respectively, with the aim to demonstrate the minor incidence of gastrointestinal side effects, revealed other serious adverse responses. Particularly, in the VIGOR study a significant increase in the risk of myocardial infarction and thrombotic events were observed in rofecoxib patients when compared with naproxen patients.

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Opioids (morphine and codeine) are used as monotherapy in the treatment of moderate to severe pain or are added to NSAIDs when the treatment with these drugs is not sufficient. Nevertheless, an overestimation of their side effects (constipation, respiratory depression, addiction and tolerance) still limits their clinical use.

In addition to NSAIDs and opioids the use of tricyclic antidepressants or anticonvulsants is quite common in neurophatic pain coupled with psychological components.

Despite the large number of drugs marketed as analgesic, it has been estimated that nearly half a billion cases of painful diseases are diagnosed each year and that over 50% of patients are unsatisfied with their present treatment [6].

Therefore researchers make every effort to find out new compounds that can more effectively and safely treat the different types of acute and chronic pain.

In the last years we focused our studies on the synthesis and pharmacological characterization of a large number of benzopyrano[4,3-d]pyrimidine derivatives, some of which are summarized in Fig. (**1**), that have supplied us with interesting information about both their chemical and biological behaviour.

The structural modifications, particularly the positions 2 and 5 of the benzopyrano[4,3-d]pyrimidine scaffold, were planned with the aim to study the antiplatelet action of the obtained molecules. In fact, nearly all the synthesized compounds showed a remarkable antiaggregant activity; many of them, such as 2,5-dicycloamino [7] or 2-methoxy-5-cycloamino derivatives [8] (see compounds **2**), showed an antiplatelet profile like aspirin, while other series, for example the 2-methylthio-5-cycloamino substituted [9] or the cou-

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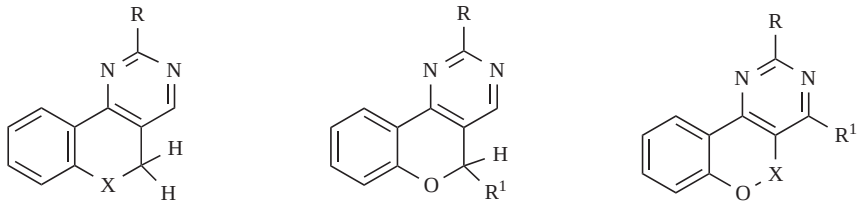


1
X = CH_2,O, S
R = $N(CH_3)CH_2COOH$
$N(CH_3)CH_2CONR"_2$
$NH(CH_2)n$-COOH
NH-$CH(CH_3)$-COOH

2
R =NH_2, cycloamines, SCH_3, OCH_3, SO_2CH_3,
R^1=cycloamines, Alkoxy

3
X = CO, CH_2
R = H, NH_2 ,cycloamines OCH_3, SCH_3 ,SO_2CH_3
R^1= H, NH_2, cycloamines

Fig. (1). Summarizing structures of previously synthesized benzopyranopyrimidines.

marin-like 2-methanesulfonyl-4-cycloamino substituted [10] (see compounds **3**), inhibited ADP or U46619-induced aggregation as well. Moreover, both 2,5-dicycloamino derivatives and some of compounds **3** showed an interesting antithrombotic activity *in vivo*, devoid of gastrolesivity and, at variance with aspirin, they did not increase bleeding [10,11].

In addition to *in vitro* antiplatelet activity, numerous benzopyranopyrimidine derivatives showed also interesting analgesic and/or antiphlogistic activity. In general, when the benzopyranopyrimidine scaffold was substituted with a methoxy or a nitrogen group (amino or aminoacid) in position 2, and with an alkoxy group in position 5, we observed the above activities, but each series showed a different pharmacological profile. For example, among compounds **1** the major part of the 2-aminoacid substituted showed antiinflammatory activity, but were devoid of analgesic one, *viceversa* the 2-amino/methoxy substituted. On the other hand, 5-alkoxy-2-methoxy-derivatives (see compounds **2**) possessed a remarkable antinociceptive activity coupled with a certain antiphlogistic action, while compounds **3** were lacking in both the activities. These compounds were less potent antiplatelet agents than the above mentioned ones but, interestingly, they were similarly lacking in ulcerogenic activity.

It was assumed that basic compounds exist in a partial ionic, lipid-insoluble form, at the low pH levels characteristic of the stomach, thus they could be less aggressive towards gastric mucosa [12]. Effectively, all our compounds having a basic function, in position 2 or 5, are lacking in ulcerogenic activity, despite their different pharmacological behaviour.

Now, because the gastrolesivity is one of the most relevant side effects for the NSAIDs, we were interested in synthesizing new basic benzopyranopyrimidines with the aim of producing more potent analgesic/antiphlogistic agents and obtaining more information about their mechanism of action.

Thus, in this work we report the synthesis of compounds **4a, 5a-f** and **6a-f** (see scheme **1**), having in position 2 three different nitrogen substituents and in position 5 the same alkoxy groups present in the analgesic/antiphlogistic compounds previously synthesized. The results of the *in vitro* and *in vivo* pharmacological study and some considerations about their structure-activity relationship are also reported.

2. CHEMISTRY

Compounds **4a**, **5a-f** and **6a-f** were prepared as reported in scheme **1**.

3-Formylchromone **7**, which was obtained from 2-hydroxyacetophenone by a Vilsmeier reaction [13], reacted with guanidine carbonate, following the Gosh procedure [14], to yield the 2-amino-5-hydroxy intermediate (**8)**. The 2-amino-5-methoxy derivative (**4a**) is prepared by refluxing compound **8** in anhydrous methanol, in presence of p-toluenesulfonic acid as a catalyst. In a previous work we demonstrated that this reaction could be a useful way to obtaining 5-alkoxy derivatives, starting from the corresponding hemiacetal, in the presence of the appropriate acid catalytic agent (HCl or H_2SO_4 or p-toluenesulfonic acid) [15].

On the other hand, 3-formylchromone **7**, upon condensation with S-methylisothiourea [16], yields the 5-hydroxy-2-methylthio intermediate **9** which, in presence of suitable alcohols and different acids as catalysts, gives the 5-alkoxy-2-methylthio derivatives (**10a-f**).

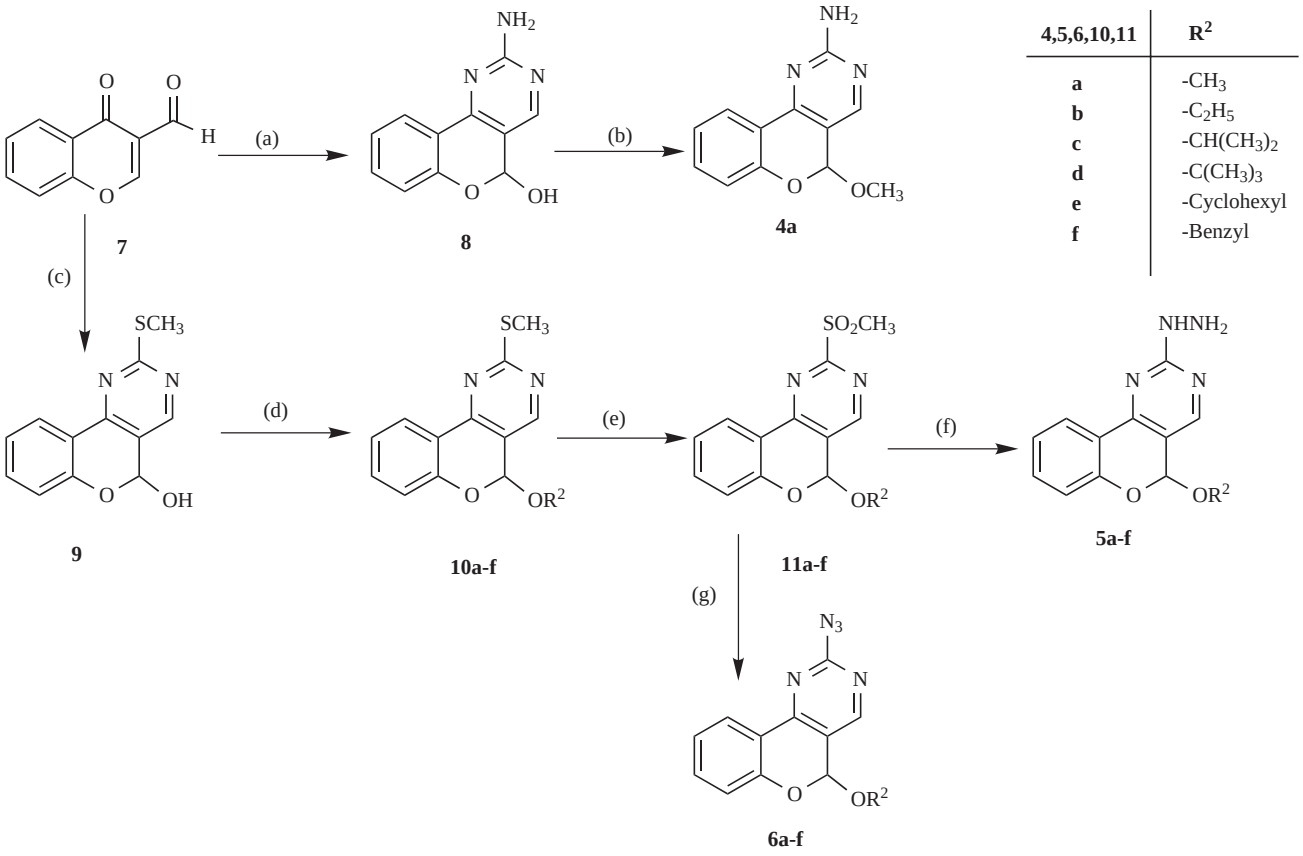
Then, the 2-methanesulfonyl derivatives (**11a-f**) were prepared from the corresponding 2-methylthio **10a-f** by oxidation with an excess of *meta*-chloroperbenzoic acid [15].

Finally, the 2-hydrazino derivatives (**5a-f**) and the 2-azido derivatives (**6a-f**) were obtained by substitution of the 2-methanesulfonyl group with hydrazine hydrate in DMSO (Dimethylsulfoxide) at 100°C and with sodium azide in DMF (N,N-Dimethylformamide) at 80°C, respectively [17].

3. PHARMACOLOGY

In vivo experiments were performed for all synthesized compounds in order to evaluate their antiphlogistic (carrageenan rat paw edema), analgesic (acetic acid induced mouse writhing test) and antipyretic [LPS (Lipopolysaccharide) induced rat fever] activities. Moreover, they were screened for their *in vitro* antiplatelet activity, in rabbit platelet-rich plasma aggregated by ADP, arachidonic acid (AA) and the stable TXA_2 (Thromboxane A_2) receptor agonist U46619.

Compounds that showed an interesting analgesic activity were then submitted to hot plate test in mice to further ascertain antinociceptive activity. Spontaneous motor activity test in mice was used to verify a possible sedative effect of the compounds.



Scheme 1. Synthesis of compounds **4a**, **5a-f** and **6a-f**:

(a) Guanidine carbonate, EtOH, reflux, 3h. (b) Anhydrous methanol, p-toluenesulfonic acid, reflux, 6h. (c) S-methylisothiourea sulfate, 1M NaOH, triethylamine 80°C, 3h. (d) Alchols, H_2SO_4 or HCl or p-toluenesulfonic acid as catalyst, reflux, 6-8h. (e) m-Chloroperbenzoic acid, $CHCl_3$, r.t., 12h. (f) NH_2-NH_2.H_2O, DMSO, 100°C, 1h. (g) Sodium azide, DMF, 80°C, 1h.

In addition, tests of gastrolesivity were performed only for the most active compounds.

Because the two series, **5** and **6**, despite the strong structural analogies, showed a different pharmacological behaviour (such as we will report in "Results and Discussion" section) we hypothesized that they could act with different mechanisms and we planned to study some selected compounds (**5a,d,e** and **6d**) on human recombinant COX 1 and COX 2.

4. RESULTS AND DISCUSSION

The 2-amino derivative (**4a**) showed only minimal antiplatelet activity *in vitro* and weak antinociceptive effect *in vivo* (Table **1** and **2**).

Pharmacological screening of the hydrazino derivatives (**5a-f**) revealed an aspirin-like profile since they inhibited AA-induced platelet aggregation, being active in the range of 34-124 μM concentrations, but failed to antagonize ADP or U46619 induced platelet aggregation up to 1 mM concentration (Table **1**).

In the enzyme assay, performed on human COX 1 and COX 2, compounds **5a,d,e** demonstrated a strong, non-selective, inhibition of cyclooxygenases [about 95% inhibition of PGE_2 (Prostaglandins E_2) production from AA by 10 μM concentration]. Compound **5d** had an IC_{50} of 54 nM towards COX1 and 60 nM towards COX 2, data comparable to those of diclofenac (IC_{50}= 14 nM) and NS398 (IC_{50}=72 nM), used as reference compounds in COX 1 and COX 2 assays, respectively. Notwithstanding the inhibition of COX activity displayed *in vitro*, compounds **5a,d,e** did not cause gastric damage in rats after acute oral administration at 200 mg/kg (data not shown).

The same derivatives exhibited good analgesic activity in the writhing test, with ED_{50} ranging from 40 to 76 mg/kg/os, with the exception of compound **5a** that was ineffective up to 100 mg/kg**.** Since analgesic doses of the compounds did not affect murine spontaneous locomotor activity, any confounding sedative effect could be ruled out (Table **2**). It has to be mentioned that among the effective compounds, derivative **5c** caused a significant prolongation of reaction time (increase of 25% at 100 mg/kg/os $p<0.05$) in the hot plate test protecting mice from thermal nociceptive stimulus. Antipyretic and antiphlogistic activity, as tested in rat LPS-induced fever and in carrageenin-induced rat paw edema, was absent for all the compounds of this series tested up to 100 mg/kg/os.

For the azido derivatives (**6a-f**) a different pharmacological behaviour was observed. In fact, only three compounds (**6a,b,c**) showed a moderate inhibitory activity in AA- and

Table 1. ***In Vitro*** **antiplatelet activity expressed as efficacy (maximal inhibition %) and potency (IC_{50}) of acetylsalicylic acid (ASA) and compounds under study on rabbit platelet rich plasma against ADP, arachidonic acid (AA) or U46619 induced aggregation (mean of 5 experiments)**

Comp.	ADP 5μM		AA 100μM		U46619 2μM	
	Maximal inhibition (%)	IC_{50} (μM)	Maximal inhibition (%)	IC_{50} (μM)	Maximal inhibition (%)	IC_{50} (μM)
4a	inactive	n.c.	40	n.c.	inactive	n.c.
5a	40	n.c.	100	39	inactive	n.c.
5b	inactive	n.c.	100	43	inactive	n.c.
5c	inactive	n.c.	100	47	inactive	n.c.
5d	inactive	n.c.	100	34	inactive	n.c.
5e	inactive	n.c.	100	124	inactive	n.c.
5f	inactive	n.c.	100	38	inactive	n.c.
6a	43	n.c.	100	447	100	641
6b	inactive	n.c.	100	534	70	613
6c	72	745	100	158	100	490
6d	inactive	n.c.	inactive	n.c.	inactive	n.c.
6e	inactive	n.c.	inactive	n.c.	inactive	n.c.
6f	inactive	n.c.	52	n.c.	inactive	n.c.
ASA	45	n.c.	100	55	inactive	n.c.

n.c. = not calculable

U46619-induced platelet aggregation; compound **6c** was the most potent and the only one active also against ADP (Table **1**). Furthermore, the results obtained for compound **6d** (10 μM) in the enzyme assays indicated complete inactivity in the inhibition of both human COX isoforms. The most remarkable differences with hydrazino series were observed *in vivo*. Compounds **6a,b,d** possessed a significant antiphlogistic activity coupled with antipyretic activity (significant only for compound **6b**). Two azido derivatives out of six (**6d,e**) showed good antinociceptive activity in the writhing test at doses devoid of sedative effects and were ineffective in the hot plate test up to 100 mg/kg.

On the whole, the hydrazino group inserted in position 2 of the benzopyranopyrimidino scaffold confers selective and potent antiplatelet activity against AA, probably due to the inhibition of COX enzyme, whereas the azido substitution is associated with a weak and aspecific antiplatelet activity.

The significant antinociceptive activity of the hydrazine derivatives resembles that shown by 2-methoxy-5-alkoxy benzopyranopyrimidines previously described [15] and it seems not to be related to the inhibition of COX isoforms. Also the considerable antiphlogistic /antipyretic activity emerged for some azido derivatives is not apparently attributable to blockade of COX 1 and COX 2.

In conclusion, we synthesized two new series of benzopyranopyrimidine derivatives, some of which showed an interesting Aspirin-like profile *in vitro* due to a strong inhibition of COX enzymes, but were devoid of gastric noxious effects. The results obtained from the tests *in vivo* suggest a different mechanism of action of the two series, and a possible decisive role of the pharmacodynamic processes.

Finally, these results are a further confirmation that the substituents in position 2 of the benzopyranopyrimidine structure are determinants for the type of pharmacological activity observed. By comparing the present findings with those obtained with the corresponding 5-alkoxy derivatives previously described [15] it seems that hydrazino but not azido substituent behaves as bioisoster group of methoxy moiety in the benzopyrano pyrimidine series. As concern the substituents in position 5 it seems reasonable to assert that the pharmacological results do not clearly indicate which group is the best one. Only in AA induced platelet aggregation it is possible to note a different level of potency of **5e** (5-cyclohexyloxy derivative) in respect to the other compounds. However, it would be necessary to synthesize a greater number of analogues for being able to assert that the cycloalkyl substituents are unfavourable for the antiplatelet activity.

5. EXPERIMENTAL SECTION

5.1. Chemistry

All chemicals were obtained from Sigma-Aldrich s.r.l. (Milan, Italy).

Table 2. Effects of compounds under study (100 mg/kg/os) on inflammatory, nociceptive, febrile responses and on spontaneous motor activity in rodents. Data are expressed as % of inhibition compared to control. In writhing test and motor activity test the compounds were administered at various doses and the inhibitory potency was indicated by ED_{50} values. Each dose was administered to 6 animals

Comp	Spontaneous motor activity	Writhing test		LPS-induced fever	Carrageenin-induced paw edema
	ED_{50} (mg/kg os)	ED_{50} (mg/kg os)	Max inhibition (%)	Max inhibition (%)	Max inhibition (%) 3hour 4 hour 5 hour
4a	n.d.	89	65%**	n.d.	0%
5a	n.d.	n.c.	27%	0%	0%
5b	Inactive	40	90%**	0%	0%
5c	Inactive	76	63%**	0%	0%
5d	Inactive	41	80%**	0%	0%
5e	81	45	70%**	0%	0%
5f	Inactive	58	92%**	0%	0%
6a	n.d.	n.c.	33%	39%	42%** 21% 39%**
6b	n.d.	n.c.	24%	64% **	37%** 26% 33%*
6c	n.d.	n.c.	0%	0%	23% 14% 22%
6d	74	35	78%**	40%	40%** 35%** 35%**
6e	Inactive	79	63%**	0%	17% 11% 7%
6f	n.d.	n.c.	40%*	13%	17% 11% 24%

n.d. = not determined

n.c. = not calculable

* P<0.05 and **P<0.01 vs vehicle treated animals (unpaired Student's t test)

Melting points are uncorrected and were measured with a Büchi 540 instrument. IR spectra were recorded with a Perkin-Elmer 398 spectrophotometer. ^{1}H-NMR spectra were recorded on a Varian Gemini 200 (200MHz) instrument; chemical shifts are reported as δ (ppm) relative to tetramethylsilane (TMS) as internal standard; signals were characterized as s (singlet), d (doublet), t (triplet), q (quartet), sept (septet), m (multiplet), br s (broad signal); *J* in Hz.

All compounds were tested for purity by TLC (Kieselgel 60F254 DC-Alufolien, E. Merck, Darmstadt, Germany).

Elemental analyses, indicated by the symbols of the elements or functions were within ±0.4% of the theoretical values and were determined with an Elemental Analyzer EA 1110 (Fison-Instruments, Milan, Italy).

5.1.1. Preparation of 2-amino-5-methoxy-5H-[1]benzopyrano[4,3-d]pyrimidine (4a)

A mixture of 2-amino-5-hydroxy-5*H*-[1]benzopyrano [4,3-d]pyrimidine (**15**) (5mmol, 1.08g) and p-toluenesulfonic acid (200mg) in an. CH_3OH (40 mL) was refluxed for 6h. The solvent was evaporated under reduced pressure and the crude solid was solved in $CHCl_3$ (25 mL); the organic solution was washed once with $NaHCO_3$ sat. solution (25 mL), once with H_2O (25 mL), then was dried ($MgSO_4$), and evaporated under reduced pressure. The white solid obtained was filtered and recrystallized from an. CH_3OH.

m.p.: 166-167 °C; yield: 67%. IR (KBr) cm^{-1}: 3335, 3333 (NH). ^{1}H NMR ($CDCl_3$) δ: 3.57 (s, 3H, OCH_3), 5.32 (br s, 2H, NH_2, disappears with D_2O), 7.05-7.20 (m, 2H, H_7+H_8), 7.47 (t, *J*=6, 1H, H_9), 8.22 (d, *J*=6, H_{10}), 8.29 (s, 1H, H_4). Anal. calcd. for $C_{12}H_{11}N_3O_2$: C, 62.87; H, 4.84; N, 18.33. Found: C, 62.83; H, 5.07; N, 18.40.

5.1.2. General Procedure for 5-alkoxy-2-hydrazino-5H-[1] benzopyrano[4,3-d]pyrimidines (5a-f).

To hydrazine monohydrate (5 mL), previously heated at 80 C°, a solution of the suitable 5-alkoxy-2-methanesulphonyl-5*H*-[1]benzopyrano[4,3-d]pyrimidine (**18a-f**) (5 mmol) in DMSO (15 mL), was added drop to drop and the reaction mixture was stirred at 100°C for 1h. After cooling, the mixture was poured into ice-water (100 mL). The yellow solid obtained was filtered and recrystallized from 95% ethanol (compounds **12a**,**d**,**e**), absolute ethanol (compounds **12b**,**f**) or absolute ethanol/$CHCl_3$ (9:1) (compound **12c**).

2-Hydrazino-5-methoxy-5H-[1]benzopyrano[4,3-d]pyrimidine (5a)

m.p. 174-175 °C; yield 99%. IR ($CHCl_3$) cm^{-1}: 3653, 3441, 3337 (NH, NH_2). ^{1}H NMR ($CDCl_3$) δ: 3.56 (br s, 5H, OCH_3+NH_2 becomes s after deuteration), 5.99 (s, 1H, H_5), 6.67 (br s, 1H, NH disappears with D_2O), 7.08-7.27 (m, 2H, H_7+H_8), 7.47 (t, *J*=6.0, 1H, H_9), 8.27 (d, *J*=6.0, 1H, H_{10}),

8.32 (s, 1H, H_4). Anal. calcd. for: $C_{12}H_{12}N_4O_2$: C, 59.01; H, 4.95; N, 22.94. Found: C, 58.73; H, 5.20; N, 22.80.

2-Hydrazino-5-ethoxy-5H-[1]benzopyrano[4,3-d]pyrimidine (5b)

m.p. 153-154°C; yield 67%. IR ($CHCl_3$) cm^{-1}: 3663, 3441, 3336 (NH, NH_2). ^1H NMR ($CDCl_3$) δ: 1.20 (t, *J*=7.0, 3H, CH_3), 3.68-4.08 (m, 4H, OCH_2+NH_2, 2H disappear with D_2O), 6.09 (s, 1H, H_5), 6.68 (br s, 1H, NH, disappears with D_2O), 7.06-7.20 (m, 2H, H_7+H_8), 7.47 (t, *J*=6.0, 1H, H_9), 8.28 (d, *J*=6.0, 1H, H_{10}), 8.31 (s, 1H, H_4). Anal. calcd. for: $C_{13}H_{14}N_4O_2$: C, 60.45; H, 5.46; N, 21.69. Found: C, 60.11; H, 5.80; N, 21.80.

2-Hydrazino-5-isopropyloxy-5H-[1]benzopyrano[4,3-d]pyrimidine (5c)

m.p. 113-115°C; yield 60%. IR ($CHCl_3$) cm^{-1}: 3668, 3441, 3335 (NH, NH_2). ^1H NMR ($CDCl_3$) δ: 1.16 (d, *J*=6.0, 3H, CH_3), 1.25 (d, *J*=6.0, 3H, CH_3), 3.92-4.18 (br s, 2H, NH_2, disappears with D_2O), 4.21-4.36 (m, 1H, OCH), 6.15 (s, 1H, H_5), 6.90 (br s, 1H, NH, disappears with D_2O), 7.02-7.20 (m, 2H, H_7+H_8), 7.44 (t, *J*=6.0, 1H, H_9), 8.20-8.32 (m, 2H, H_{10}+H_4). Anal. calcd. for: $C_{14}H_{16}N_4O_2$: C, 61.75; H, 5.92; N, 20.57. Found: C, 61.44; H, 5.92; N, 20.87.

2-Hydrazino-5-(tert-butyloxy)-5H-[1]benzopyrano[4,3-d]pyrimidine (5d)

m.p. 149-150°C; yield 85%. IR ($CHCl_3$) cm^{-1}: 3670, 3440, 3335 (NH, NH_2).^1H NMR ($CDCl_3$) δ: 1.39 (s, 9H, $3CH_3$), 6.37 (s, 1H, H_5), 6.61 (br s., 1H, NH disappears with D_2O), 7.02 (d, *J*=6.0, 1H, H_7), 7.13 (t, *J*=6.0, 1H, H_8), 7.44 (t, *J*=6.0, 1H, H_9), 8.20-8.29 (m, 2H, H_4+H_{10}). Anal. calcd. for $C_{15}H_{18}N_4O_2$: C, 62.92; H, 6.34; N, 19.57. Found: C, 62.72; H, 6.25; N, 19.27.

2-Hydrazino-5-cyclohexyloxy-5H-[1]benzopyrano[4,3-d]pyrimidine (5e)

m.p. 120-121 °C; yield 74%. IR ($CHCl_3$) cm^{-1}: 3670, 3441, 3334 (NH, NH_2).^1H NMR ($CDCl_3$) δ: 1.28-2.10 (m, 10H, $5CH_2$ cyclohex.), 3.87-4.04 (m, 2H, OCH +NH, 1H disappears with D_2O), 6.24 (s, 1H, H_5), 6.52 (br s, 1H, NH, disappears with D_2O), 7.05-7.16 (m, 2H, H_7+H_8), 7.45 (t, *J*=6.0, 1H, H_9), 8.25-8.30 (m, 2H, H_{10}+H_4). Anal. calcd. for $C_{17}H_{20}N_4O_2$: C, 65.37; H, 6.45; N, 17.94. Found: C, 65.25; H, 6.75; N, 17.68.

2-Hydrazino-5-benzyloxy-5H-[1]benzopyrano[4,3-d]pyrimidine (5f)

m.p. 123-124°C; yield 50%. IR ($CHCl_3$) cm^{-1}: 3676, 3441, 3335 (NH, NH_2).^1H NMR ($CDCl_3$) δ: 3.97-4.18 (br s, 2H, NH_2 disappears with D_2O), 4.83 and 4.89 (AB q, *J*=13.0, 2H, OCH_2), 6.15 (s, 1H, H_5), 6.73 (br s, 1H, NH, disappears with D_2O), 7.08 (d, *J*=6.0, 1H, H_7), 7.17 (t, *J*=6.0, 1H, H_8), 7.28-7.39 (m, 5H, Ar), 7.46 (t, *J*=6.0, 1H, H_9), 8.23-8.33 (m, 2H, H_{10}+H_4). Anal. calcd. for $C_{18}H_{16}N_4O_2$: C, 67.49; H, 5.03; N, 17.49. Found: C, 67.29; H, 5.29; N, 17.69.

5.1.3. General procedure for 2-azido-5-alkoxy-5H-[1]benzopyrano[4,3-d]pyrimidines (6a-f).

To a suspension of the suitable 5-alkoxy-2-methanesulphonyl-5*H*-[1]benzopyrano[4,3-d]pyrimidine (**12a-f**) (5 mmol) in an. DMF (20 mL), NaN_3 (3 g, 46 mmol) was added and the mixture stirred at 80°C for 1h. After cooling, the reaction mixture was poured into ice-water (100 mL); the brown amorphous solid precipitated was filtered and purified by flash chromatography with Florisil® (100-200 mesh) and CH_2Cl_2 as eluent. The light yellow solids obtained were recrystallized from absolute ethanol (compounds **6a-c**) or absolute ethanol/$CHCl_3$ (9:1) (compounds **6d-f**).

2-Azido-5-methoxy-5H-[1]benzopyrano[4,3-d]pyrimidine (6a)

m.p. 139-141 °C; yield 60%. IR ($CHCl_3$) cm^{-1}: 2142 (N_3). ^1H NMR ($CDCl_3$) δ: 3.58 (s, 3H, OCH_3), 6.07 (s, 1H, H_5), 7.02-7.61 (m, 3H, H_7+H_8+H_9), 8.34 (d, *J*=6.0, 1H, H_{10}), 8.53 (s, 1H, H_4). Anal. for $C_{12}H_9N_5O_2$: C, 56.47; H, 3.55; N, 27.44. Found: C, 56.08; H, 3.77; N, 27.20.

2-Azido-5-ethoxy-5H-[1]benzopyrano[4,3-d]pyrimidine (6b)

m.p. 112-113°C; yield 84%. IR ($CHCl_3$) cm^{-1}: 2150 (N_3). ^1H NMR ($CDCl_3$) δ: 1.22 (t, *J*=7.0, 3H, CH_3), 3.73-4.10 (m, 2H, OCH_2), 6.20 (s, 1H, H_5), 7.08-7.28 (m, 2H, H_7+H_8), 7.51 (t, *J*=6.0, 1H, H_9), 8.32 (d, *J*=6.0, 1H, H_{10}), 8.51 (s, 1H, H_4). Anal. for $C_{13}H_{11}N_5O_2$: C, 57.99; H, 4.12; N, 26.01. Found: C, 57.59; H, 4.14; N, 25.95.

2-Azido-5-isopropyloxy-5H-[1]benzopyrano[4,3-d]pyrimidine (6c)

m.p. 110-111°C; yield 56%. IR ($CHCl_3$) cm^{-1}: 2130 (N_3). ^1H NMR ($CDCl_3$) δ: 1.20 (d, *J*=6.0, 3H, CH_3), 1.29 (d, *J*=6.0, 3H, CH_3), 4.29 (sept, *J*=6.0, 1H, OCH), 6.26 (s, 1H, H_5), 7.05-7.30 (m, 2H, H_7+H_8), 7.50 (t, *J*=6.0, 1H, H_9), 8.32 (d, *J*=6.0, 1H, H_{10}), 8.47 (s, 1H, H_4). Anal. for $C_{14}H_{13}N_5O_2$: C, 59.36; H, 4.63; N, 24.72. Found: C, 59.60; H, 4.67; N, 24.89.

2-Azido-5-(tert-butyloxy)-5H-[1]benzopyrano[4,3-d]pyrimidine (6d)

m.p. 127-128°C; yield 89%. IR ($CHCl_3$) cm^{-1}: 2142 (N_3). ^1H NMR ($CDCl_3$) δ: 1.36 (s, 9H, $3CH_3$), 6.45 (s, 1H, H_5), 7.03 (d, *J*=6.0, 1H, H_7), 7.17 (t, *J*=6.0, 1H, H_8), 7.48 (t, *J*=6.0, 1H, H_9), 8.30 (d, *J*=6.0, 1H, H_{10}), 8.41 (s, 1H, H_4). Anal. for $C_{15}H_{15}N_5O_2$: C, 60.60; H, 5.09; N, 23.56. Found: C, 60.42; H, 5.00; N, 23.80.

2-Azido-5-cyclohexyloxy-5H-[1]benzopyrano[4,3-d]pyrimidine (6e)

m.p. 140-141 °C; yield 79%. IR ($CHCl_3$) cm^{-1}: 2143 (N_3). ^1H NMR ($CDCl_3$) δ: 1.18-2.11 (m, 10H, $5CH_2$ cycloex), 3.90-4.11 (m, 1H, OCH), 6.29 (s, 1H, H_5), 7.06-7.33 (m, 2H, H_7+H_8), 7.50 (t, *J*=6.0, 1H, H_9), 8.31 (d, *J*=6.0, 1H, H_{10}), 8.47 (s, 1H, H_4). Anal. for $C_{17}H_{17}N_5O_2$: C, 63.15; H, 5.30; N, 21.66. Found: C, 62.92; H, 5.40; N, 21.87.

2-Azido-5-benzyloxy-5H-[1]benzopyrano[4,3-d]pyrimidine (6f)

m.p. 129-130 °C; yield 69%. IR ($CHCl_3$) cm^{-1}: 2140 (N_3). ^1H NMR ($CDCl_3$) δ: 4.84 and 4.89 (AB q, *J*=12.0, 2H, O-CH_2), 6.23 (s, 1H, H_5), 7.04-7.40 (m, 7H, H_7+H_8+5HAr), 7.50 (t, *J*=6.0, 1H, H_9), 8.33 (d, *J*=6.0, 1H, H_{10}), 8.44 (s, 1H, H_4). Anal. for $C_{18}H_{13}N_5O_2$: C, 65.25; H, 3.95; N, 21.14. Found: C, 65.00; H, 3.70; N, 21.10.

5.2. Materials and pharmacological methods.

5.2.1. Animals

Swiss mice, Wistar rats and rabbits were used for the experiments. The animals were fasted at 20°C for 24 hours with free access to water. All the experiments were performed according to ethical standard guidelines and were approved by Italian Ministry of Health (DL 116/92).

5.2.2. In Vitro Experiments

Platelet Aggregation Assay

PRP from rabbits was used to perform aggregation in the Aggrecorder PA 3220 (Menarini, Firenze) following Born's turbidimetric method [18]. Aggregation was recorded as the percent change in light transmission: the baseline value was set using PRP and maximal transmission using PPP. PRP was preincubated at 37°C for 5 min with solvent (dimethyl sulfoxide, DMSO, final concentration 0.5%) or the compounds under study (1-1000 μM) before the addition of the platelet agonists. Maximal aggregation was obtained stimulating platelets with 5 μM ADP, 100 μM arachidonic acid or 2 μM U46619, a stable Thromboxane A_2 agonist. Tests were performed within 3 h to avoid platelet inactivation. The effects of compounds and acetylsalicylic acid (reference drug) were expressed as percent inhibition compared with control samples containing the different aggregator agents. 0.5% DMSO did not interfere with platelet aggregation.

5.2.3. In Vivo Experiments

Analgesic, Antiphlogistic, Antipyretic Activities

Test compounds were suspended in 0.5% methoxycellulose and orally administered (100 mg/kg) to animals 1 hour before the application of agents inducing inflammatory, nociceptive or febrile responses [15]. Briefly, antiphlogistic activity was studied in rats inducing paw edema by mean of carrageenin (1%) sub plantar injection (0,1 mL). Analgesic activity was evaluated in mice using acetic acid writhing test (0.2 mL 0.6% i.p.) and antipyretic activity was determined in rats with E.Coli LPS (100 μg/kg 0.2mL i.p.) induced fever. Pharmacological activities were expressed as percentage of inhibition calculated from the difference in the response between treated and vehicle-treated group at the time of maximum noxious effect. To evaluate analgesic potency the compounds were administered at various doses. Dose-response curves were constructed to evaluate antinociceptive effect in order to obtain the ED_{50}, the dose that produced 50% of antinociception (50% reduction of control writes).

The hot plate test was performed according to the method described by Eddy and Leinbach [19]. Mice were individually placed on the 55°C hot plate apparatus (Model 475, Technical Lab Instruments Inc, Pequannock (NJ), USA) and the time between the placement and the occurrence of anterior paw licking, shaking or jumping was recorded as Latency Time (s). In order to exclude hypo- or hyper- sensitive mice, two hours before the final experiment all the animals were tested and those with latency time shorter than 10 sec or longer than 18 sec were eliminated from the study. Basal Latency Time (T0) was measured before the administration of drugs or vehicle. Forward Latency Times (T1) were measured starting 1 hour after oral treatment at intervals of 15, 30 and 60 min. Time of 30 sec was arbitrarily chosen as cut-off time (T2). Results were expressed as percentage of analgesic effect as follows: % MPE (percent maximal possible effect) = (T1 – T0)/(T2 – T0) x 100 (latency time after treatment – basal latency time)/(time of cut off – basal latency time).

Locomotor Activity

Locomotor activity was measured by means of an activity cage (height 35 cm, width 23 cm, depth 19 cm, Model 7401, Ugo Basile, Comerio (VA), Italy). One hour after oral administration of compounds (100 mg/kg) or vehicle, mice were placed singularly into the activity cage and locomotor activity was recorded at interval times of 5 minutes for 90 min. All experiments were conducted from 9.00 to 13.00.

Acute Gastrointestinal Ulcerogenicity

Acute gastrointestinal ulcerogenicity was assessed in rats treated orally with 200 mg/kg of compounds. After 5 hours, animals were sacrificed by CO_2 inhalation, the stomachs were removed, fixed in 4% formaldehyde solution and processed for microscopic analysis using an image analyzer system (Leitz, ASM 68K). The total damaged area (mm^2) and the number of gastric ulcers were counted for each stomach by an observer unaware of the treatment given to the animals.

Statistical Analysis

Results were expressed as mean±SEM. The potency of the compounds in inhibiting platelet aggregation is indicated as IC_{50} value (the concentration required to halve the maximal agonist response). This value is calculated through linear regression analysis of the inhibitory concentration-response curve constructed for each compound. Every point of the concentration-response curves was obtained from 4 independent observations. Statistical analysis was performed using two-tailed Student's t-test for paired or unpaired data. $p<0.05$ was considered significant, $p<0.01$ was considered very significant.

5.2.4. Enzyme Assay

Compounds **5a,d,e** and **6d** were tested in the *in vitro* human recombinant COX 1 and COX 2 assay (Sf9 cells), in presence of diclofenac and NS398 as reference compounds following the method reported from Glaser *et al*. [20] as described in Cerep web site (www.Cerep.fr).

ACKNOWLEDGMENTS

The skilful help of Dr G. Domenichini, Dr R. Raggio, Mr O. Gagliardo and Mr F. Tuberoni in the experimental work is gratefully acknowledged. We also thank the CEREP institute (Le Bois l'Evêque, France) for the COX_1/COX_2 studies performed under the supervision of the study director Dr. Gervais Neliat and MIUR (Ministero Italiano Università e Ricerca) for financial support.

ABBREVIATION

AA = Arachidonic acid

an = Anhydrous

Anal. calcd	=	Analyses calculated
CLASS	=	Celecoxib Long-term Arthritis Safety Study
DMF	=	N,N-Dimethylformamide
DMSO	=	Dimethylsulfoxide
LPS	=	LipoPolySaccharide
NSAIDs	=	Non Steroidal Anti-Inflammatory Drugs
PGE_2	=	Prostaglandins E_2
sat	=	saturated
TXA_2	=	Thromboxane A_2
VIGOR	=	Vioxx Gastrointestinal Outcomes Research

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Received: 13 July, 2006 **Revised: 19 September, 2006** **Accepted: 20 September, 2006**