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New class of potent antinociceptive and antiplatelet 10*H*-phenothiazine-1-acylhydrazone derivatives

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Abstract—In this work, we reported the synthesis and evaluation of the analgesic, antiinflammatory, and antiplatelet properties of new phenothiazine-attached acylhydrazone derivatives (**6**), designed exploring the molecular hybridization approach between antipsychotic chlorpromazine (**4**) and other heterocyclic derivatives (**3**) and (**5**) developed at LASSBio. Target compounds were synthesized in very good yields exploiting diphenylamine (**7**) as starting material, through regioselective functionalization of the C-1 position of 10*H*-phenothiazine ring. The evaluation of platelet antiaggregating profile lead us to identify a new potent prototype of antiplatelet derivative, that is (**6a**) ($IC_{50} = 2.3\ \mu M$), which acts in arachidonic acid pathway probably by inhibition of platelet COX-1 enzyme. Additionally, the change of *para*-substituent group of acylhydrazone framework permitted us to identify hydrophilic carboxylate derivative (**6g**) and hydrophobic bromo derivative (**6b**) as two new leads of analgesics more active than dipyrone used as standard and with selective peripheral or central mechanism of action.

1. Introduction

Hydrazone (B, Fig. 1) and acylhydrazone (C, Fig. 1) moieties are the most important pharmacoforic cores of several antiinflammatory, antinociceptive, and antiplatelet derivatives developed at LASSBio.[1] The biological profile of compounds presenting this subunit are related to its relative acidity and its capacity to stabilize free radicals, mimicking bis-allyl fragment (A, Fig. 1) of certain unsaturated fatty acids, for example, arachidonic acid (**1**), contributing to inhibit the active site of oxidative catabolic enzymes cyclooxygenase (COX) and/or 5-lypooxygenase, which are responsible for the biosynthesis of prostaglandins, thromboxanes, and leukotrienes.[2] The role of these autacoids in the genesis of several pathological states, including inflammation, pain, and asthma, are well known and decurrent from the over-expression of an induced isoform of COX, named COX-2.[3] In fact, selective COX-2 inhibitors comes being one of the most useful and successful classes of antiinflammatory drugs, due to its therapeutical safety and efficacy.[4] On the other hand, the action of certain heterocyclic hydrazone[5] and acylhydrazone[6] derivatives on blood platelets avoid platelet aggregation phenomena induced by (**1**) probably due to the inhibition of COX-1 and the consequent reduction of the bioformation of thromboxane A_2, as showed by pyrazolyl-4-acylhydrazone derivative (**3**) ($IC_{50} = 23.7\ \mu M$).[7] In spite of the discovery of that two isoforms of COX, the mechanism of action of classical analgesics and

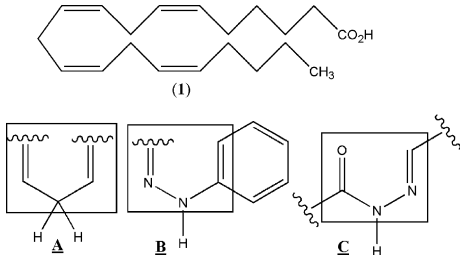

Figure 1. Hydrazone (B) and acylhydrazone (C) groups as mimicks of bis-allyl fragment (A) of arachidonic acid (**1**).

Keywords: 10*H*-phenothiazine; Acylhydrazone derivatives; Analgesic activity; Antiplatelet drugs; Molecular hybridization.
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antipyretic drugs have remained unclear until the work of Chandrasekharan et al.[8] indicated the existence of a third isoform of COX derived from COX-1 gene, that is COX-3, which occurs mainly in the cerebral cortex and are selectively inhibited by paracetamol and dipyrone.

Considering this panorama, we decided to construct a new class of 10*H*-phenothiazine-1-acylhydrazone derivatives as attractive candidates to antinociceptive and antiplatelet agents, designed by molecular hybridization between antipsychotic drug chlorpromazine (**4**) and acylhydrazones (**3**) and (**5**), which presented, respectively, important platelet anti-aggregating[7] and analgesic[9] properties. The design concept of these compounds explored the introduction of acylhydrazone moiety (**b**, Fig. 2) in the neurotropic phenothiazine framework (**a**, Fig. 2) aiming a selective action at CNS level, hoping through COX-3 modulation of. The position of the heterocyclic ring elected for the anchoration of acylhydrazone group was at C-1 due to the conformational restriction imposed by the six-membered intramolecular hydrogen bonding between N–H of phenothiazine ring and carbonyl group of acylhydrazone subunit (Fig. 2). The nature of the substituent *W* present in the phenyl group of subunit *Ar* of the derivatives (**6**), that is 4-H, 4-Br, 4-OMe, 4-NO_2, 4-$N(CH_3)_2$, 4-CO_2H and 3,5-ditertBu-4-OH, was elected in order to introduce in this series of compounds an important electronic variation[10] [σ_p-Hammett values ranging from −0.83 (NMe_2) to +0.78 (NO_2)], which could be useful to investigate the contribution of this structural sub-unit on its bioactivity profile. On the other hand, considering the importance of the presence of the furyl group in aza-arylidene moiety for the antiplatelet activity as evidenced in the previous series,[5] we decided to investigate the contribution to the antiinflammatory, analgesic, and antiplatelet activity of the substitution of this pharmacophoric group by isosteric 2-thiophenyl ring in the subunit *Ar* producing the derivatives (**6h–i**).

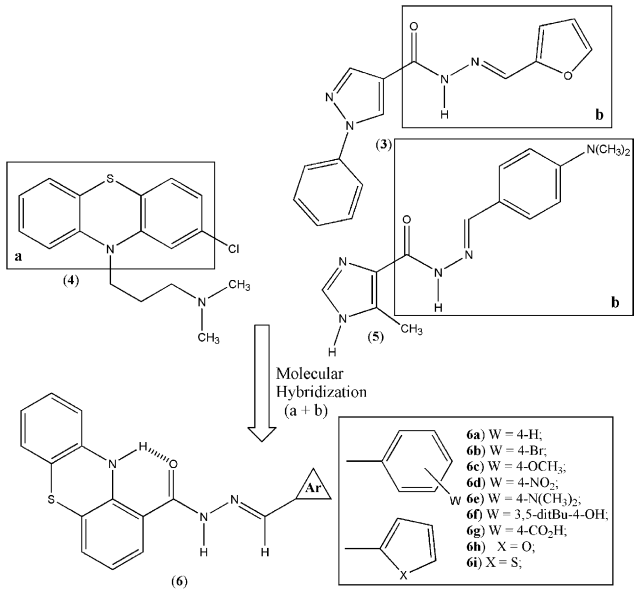
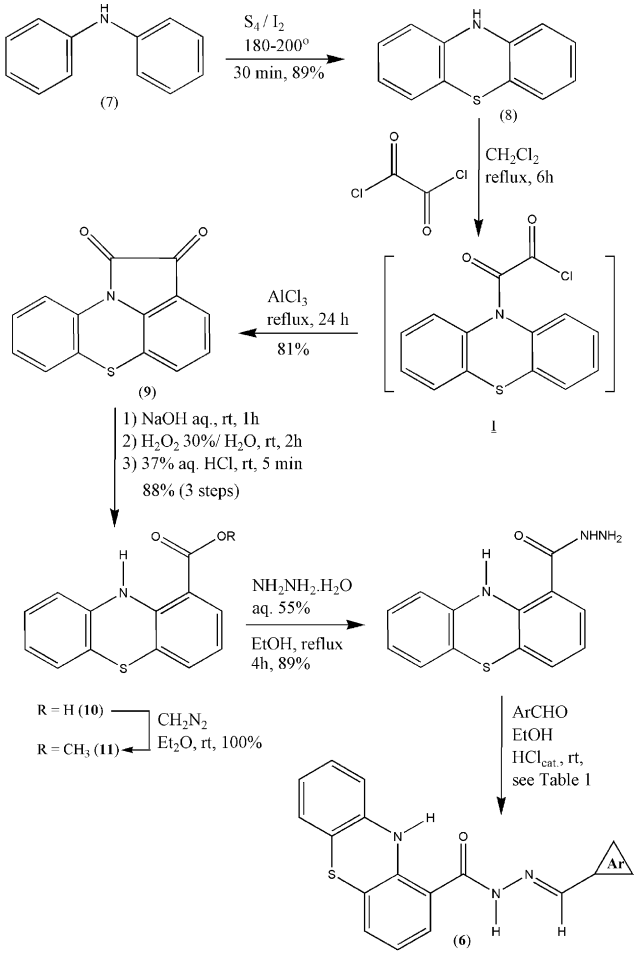


Figure 2. Design concept of new 10*H*-phenothiazine-1-acylhydrazone derivatives (**6a–i**).

2. Chemistry

The new substituted 10*H*-phenothiazine-1-acylhydrazone derivatives (**6a–i**) were synthesized exploring diphenylamine (**7**) as starting material to the preparation of the heterocyclic phenothiazine ring (**8**) in 89% yield, through the classical condensation by fusion with powder sulfur[11] (Scheme 1). Next, to achieve the regioselective functionalization of the position 1 of (**8**) we decided to proceed the construction of the tetracyclic isatin intermediate (**9**) involving the neighbor assistance of the *ortho*-nitrogenated group. In fact, compound (**9**) was obtained in 81% yield using an 'one-pot' two steps procedure previously described by Boekelheide and Hollins[12] and modified by Lopes et al.[13], which consisted in the *N*-acylation of the nitrogen atom of (**8**) by treatment with oxalyl chloride in dichloromethane in reflux, followed by Friedel–Crafts cyclization of the monooxalylamide intermediate *I* before the addition of anhydrous aluminum chloride (Scheme 1). The isatin derivative (**9**) was converted in 88 % yield to 10*H*-phenothiazine-1-carboxylic acid (**10**) after basic hydrolysis of amide bond by it treatment with an aqueous solution of sodium hydroxide at room temperature, followed by oxidative cleavage of the α-ketoacid intermediate mediated by addition of 30% aq hydrogen peroxide solution



Scheme 1. Synthetic route for the preparation of phenothiazine NAH derivatives (**6a–i**).

(Scheme 1). The derivative (**10**) was quantitatively converted into corresponding methyl ester derivative (**11**) by treatment with an ethereal solution of diazomethane at room temperature.[12]

The key acylhydrazine intermediate (**12**) was obtained in 70% yield by refluxing an ethanolic solution of the methyl ester derivative (**12**) with hydrazine hydrate for 4 h (Scheme 1).[14,15]

Finally, the new NAH target compounds (**6**) were obtained, in very good yields, by condensing compound (**12**) with the corresponding aromatic aldehydes (Ar-CHO) in ethanol, using hydrochloric acid as catalyst[14,15] (Scheme 1, Table 1).

The analysis of ^{1}H NMR spectra and HPLC chromatograms (Table 1) of the obtained acylhydrazones (**6a–i**) indicated that the condensation reaction was highly diastereoselective since only one diasteomer at imine double bond level was evidenced in both methods. Trying to characterize unambiguously the geometry of the diastereomer synthesized we elected the NOE experiment involving the analysis of the relationship between the irradiation of imine hydrogen and the intensity of hydrogen attached to the peptide bond as a tool enable to define the relative configuration of compounds (**6a–i**). In order to assure that conformational flexibility of these compounds may not mask the results from NMR experiments we performed molecular modeling studies using semiempirical AM1 method[16] to obtain some information about the conformational arrangement of the new target compounds. After geometry optimizations the heat of formation of the main conformations of diastereomers (*E*) and (*Z*) of compound (**6a**) were obtained as showed in Table 2.

In spite of the similar energy profile of these conformations we are able to evidence the clear preference for the conformations *S-cis* where the intramolecular hydrogen between heterocyclic N–H and the carbonyl group making a six-membered ring, in agreement with previously results from literature to phenothiazine derivatives functionalized in C-1 with carbonyl group.[17] Additionally it is possible to identify a slight preference of the *syn* conformations between carbonyl and N–H amide groups of (**6a**) (Table 2).

These results seem to indicate that analysis of NOE experiments could really express the configuration at imine double bond due to the relative rigidity of acylhydrazone side chain. Thus, after carrying out the double irradiation of the N–H amide signal at δ 12.0 and the imine hydrogen signal at δ 8.3 of the *para*-bromophenyl derivative (**6b**) we were able to characterize a cross-correlation with each other with the intensity of 25% and 18%, respectively (Fig. 3), confirming that the acylhydrazone derivatives obtained herein presented the relative (E) configuration. These results were extended for all structurally correlated derivatives, since they presented the same spectroscopic pattern in ^{1}H and ^{13}C NMR (Table 3) and other common spectroscopic methods (see Experimental section).

3. Results and discussion

The analgesic and antiinflammatory activities of the 10*H*-phenothiazine-1-acylhydrazone derivatives (**6a–i**) were evaluated using, respectively, the carrageenan-induced rat paw edema test[18] and the classical acetic acid-induced mice abdominal constrictions test,[19] *p.o.*, with indomethacin and dipyrone as standards. The results are disclosed in Tables 4 and 5, respectively.

Only compounds (**6e**) and (**6g**) presenting 4-dimethylamino and 4-carboxy groups were able to inhibit significantly the formation of edema in 20.5% and 51.2%, respectively (Table 4). Among all compounds tested only the acidic one, (**6g**), demonstrated some gastric irritability. This profile could be due to the inhibition of COX-1 in function of the introduction of the pharmacophoric carboxyl group present in nonselective COX inhibitors.

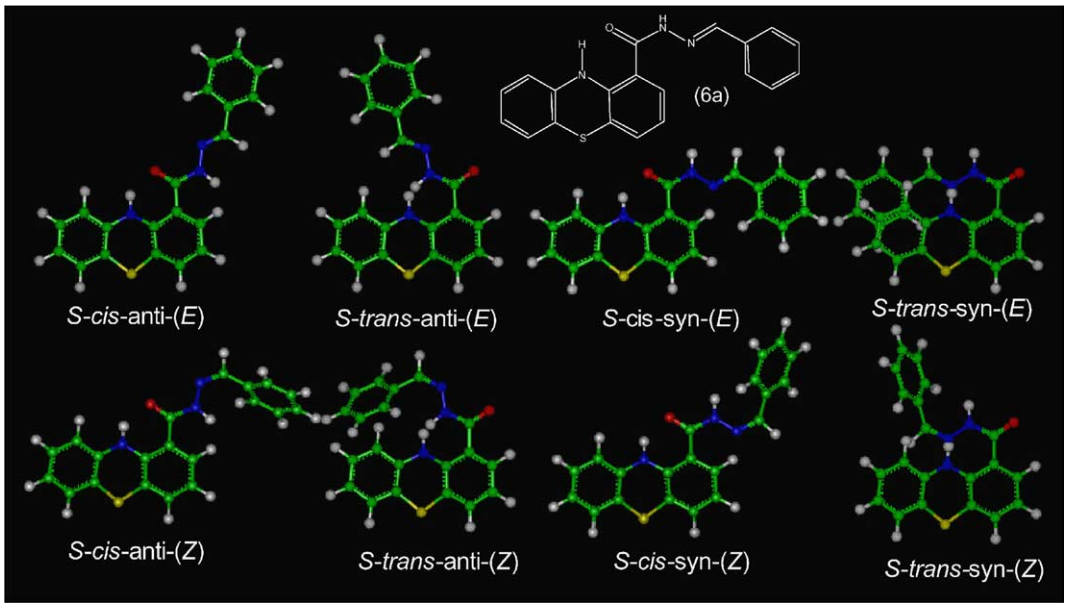
In view to evaluate the 5-LO profile for these derivatives compounds (**6e**) and (**6g**) were assayed on the pleurisy induced by carrageenan (500 μM/cavity).[20] None of

Table 1. Physical and spectral properties of the 10*H*-phenothiazine-1-acylhydrazone derivatives (**6a–i**)

Compound	Molecular formula[a]	Molecular weight	Yield (%)	M.p. (°C)	R_f[b] (min)	δ (ppm)[c]	
						N=C*H*	N–H
6a	$C_{20}H_{15}N_3OS$	345.09	97	202	4.54	8.3	12.0
6b	$C_{20}H_{14}BrN_3OS$	423.00	97	235	5.76	8.3	12.0
6c	$C_{20}H_{14}N_4O_3S$	390.08	94	272	4.87	8.4	12.0
6d	$C_{21}H_{15}N_3O_3S$	389.08	90	>250	10.01	8.3	12.0
6e	$C_{22}H_{20}N_4OS$	388.14	92	218	4.98	8.3	11.9
6f	$C_{21}H_{17}N_3O_2S$	375.10	96	216	4.64	8.3	11.9
6g	$C_{28}H_{31}N_3O_2S$	473.21	90	228	6.50	8.3	9.9
6h	$C_{18}H_{13}N_3O_2S$	335.07	97	215	3.82	8.5	12.0
6i	$C_{18}H_{13}N_3OS_2$	351.05	96	215	4.10	8.4	12.0

[a] The analytical results for C, H, N, S were within ±0.4% of calculated values.
[b] Retention factor was determined by HPLC by using a Rexchrom 5 μm RP-18 column (125×4.6 mm) and a mixture of methanol–water (7:3 v/v) as eluent at flow rate of 1 mL/min.
[c] Data obtained at 200 Mz, using DMSO-d_6 as solvent.

Table 2. Minimum energy conformers of 10*H*-phenothiazine-1-acylhydrazone derivative (**6a**)



Conformer	AM1 ΔH_f (kcal/mol)	*S-trans*/*S-cis* Conformer $\Delta\Delta H_f$ (kcal/mol)
S-cis-anti-(*E*)	98.44	1.65
S-trans-anti-(*E*)	100.09	
S-cis-syn-(*E*)	96.84	1.94
S-trans-syn-(*E*)	98.78	
S-cis-anti-(*Z*)	95.10	1.83
S-trans-anti-(*Z*)	96.93	
S-cis-syn-(*Z*)	93.62	2.01
S-trans-syn-(*Z*)	95.63	

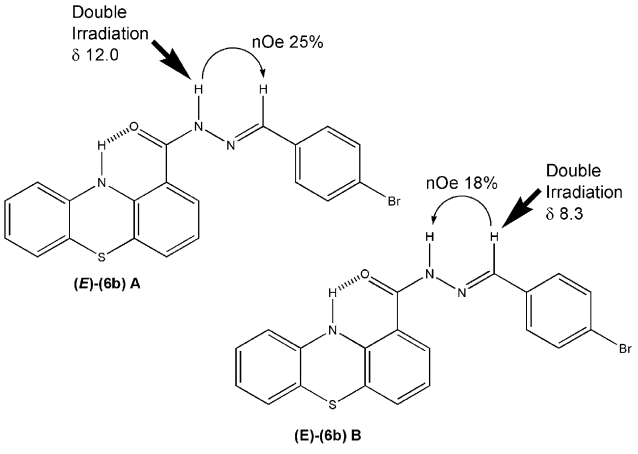


Figure 3. NOE correlations between carbonyl N–H bond and benzylidene hydrogen of the *para*-bromo derivative (**6b**).

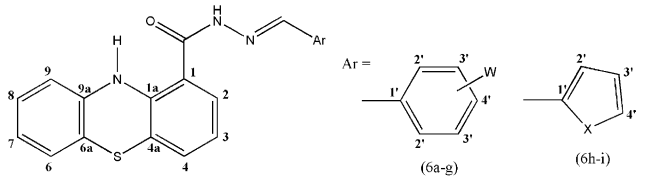
these compounds were able to inhibit the cell migration and the exudates formation (data not shown).

In spite of poor antiinflammatory profile of most of the 10*H*-phenothiazine-1-acylhydrazone derivatives (**6**) and acylhydrazide intermediate (**12**), all of them inhibited significantly the constrictions induced by acetic acid in a range from 23% to 70% (Table 5). The most active compounds were isosteric 4-nitro (**6d**) and 4-carboxy (**6g**) derivatives with 68% and 69.4% of inhibition of induced contortions, respectively, higher than dipyrone used as standard (35.9%).

Since compounds possessing a phenothiazine ring, for example, chlorpromazine (**4**), present some actions on the CNS, we have also investigated the central analgesic activity for the phenothiazine-1-acylhydrazone series on the hot plate test[21] using morphine as a reference of the experimental protocol. Only compounds *para*-Br (**6b**) and *para*-OCH_3 (**6c**) increased expressively the latency time index (LTI) in 26% and 17%, respectively, 2 h after its *p.o.* administration (Chart 1). Compound (**6b**), which is one of those derivatives of this series that presents the greater calculated log *P* value,[22] showed to be twofold more active than dipyrone used as standard (LTI = 10%). The good central analgesic profile of this particular compound could be due to it lipophylic character that favor the blood–brain barrier crossing promoting a possible COX-3 inhibition.

Additionally, these new derivatives (**6a**–**i**) were next screened in order to evaluate their effects on in vitro rabbit platelet aggregation[23] induced by arachidonic

Table 3. ^{13}C NMR data at 50 MHz ($CDCl_3$) of substituted 10*H*-phenothiazine-1-acylhydrazone derivatives **6a–i**



Carbon	Compounds								
	6a	**6b**	**6c**	**6d**	**6e**	**6f**	**6g**	**6h**	**6i**
C-1	115.19	115.26	115.57	115.03	115.87	115.63	115.57	115.33	116.64
C-2	127.66	127.85	128.06	127.86	128.10	127.85	128.06	127.80	127.71
C-3	120.27	120.81	121.05	120.81	121.19	120.83	121.05	120.77	120.55
C-4	128.25	129.75	129.80	129.92	129.69	129.50	129.80	129.60	127.71
C-4a	120.37	118.92	119.04	118.99	118.96	118.78	119.04	118.90	118.32
C-6a	117.34	116.69	116.84	116.68	116.87	116.63	116.84	116.65	116.82
C-6	126.14	126.91	126.97	126.26	126.50	126.24	126.97	126.21	122.69
C-7	122.16	123.07	123.24	123.13	123.24	123.99	123.24	123.00	127.27
C-8	128.52	126.25	126.45	126.26	127.04	126.83	126.45	126.76	115.52
C-9	116.69	115.81	115.90	115.87	115.72	115.77	115.90	115.69	139.40
C-9a	141.70	140.36	140.66	140.56	140.43	140.49	140.56	140.78	140.32
C-1a	143.09	143.25	143.36	143.35	143.31	143.14	143.35	142.97	143.09
C=N	147.75	147.21	149.83	148.72	145.83	149.91	147.42	146.31	145.31
C-1′	135.94	133.43	121.46	126.79	140.43	139.12	126.79	143.08	149.30
C-2′	127.01	131.87	128.84	129.03	128.84	125.28	129.03	128.93	113.66
C-3′	128.52	128.98	111.95	111.95	124.06	122.98	111.95	128.45	112.19
C-4′	128.84	123.49	151.82	151.82	147.02	156.43	147.02	130.25	138.14
*C*ONH	167.46	164.47	164.21	164.44	164.70	164.02	164.73	165.06	164.23
CO_2H	—	—	—	—	—		167.02		
O*C*H_3	—	—	—	55.46	—		—		
CH_3	—	—	39.18	—	—	30.14	—		

Table 4. Antiinflammatory activity of 10*H*-phenothiazine-acylhydrazone derivatives (**6a–i**) and acylhydrazide intermediate (**12**)

Compound	W or X	*n*	Volume variation (mL)	Inhibition (%)
Vehicle Control	—	5	526.6 ± 26.2	—
Indomethacin	—	5	128.4 ± 19.9	76.0*
6a	W = 4-H	11	464.2 ± 18.1	11.9
6b	W = Br	9	573.5 ± 69.3	−8.9
6c	W = 4-OCH_3	9	501.1 ± 18.1	4.8
6d	W = NO_2	10	531.0 ± 46.2	−0.8
6e	W = 4-N(CH_3)$_2$	12	418.9 ± 23.8	20.5*
6f	W = 3,5-di-tertbutyl-4-OH	12	476.7 ± 42.6	9.5
6g	W = COOH	5	361.3 ± 51.2	51.2*
6h	X = O	6	494.9 ± 27.9	6.0
6i	X = S	8	469.0 ± 42.6	10.9
12	–	6	602.7 ± 43.0	−14.4

All compounds were administered *p.o.* at a dose of 100 μmol/kg % of inhibition obtained by comparison with vehicle control group. Results are expressed as mean ± SEM. *n* = number of animals.
* $p < 0.05$ (Student's '*t*' test).

acid (AA, 100 μM), collagen (5 μg/mL) or adenosine 5-diphosphate (ADP, 5 μg/mL) (Table 6).

All test compounds inhibited by 100% the collagen- and AA-induced platelet aggregation, at 100 μM concentration, without interference on the ADP-induced aggregation. These results suggest strongly a possible modulation of enzymes or metabolites of the AA cascade.

Hydrazide intermediate (**12**) was able to inhibit completely the second wave of platelet aggregation induced by ADP in human PRP. In citrated plasma this second wave is mediated by the TXA_2 and it is abolished by indomethacin or aspirin, corroborating with a possible modulation of AA cascade derivatives, probably through inhibition of the COX-1 enzyme.

Some authors have described an antiplatelet activity for chlorpromazine on the collagen-induced platelet aggregation.[24,25] In our experiments, chlorpromazine at 100 mM concentration did not inhibit the AA-induced aggregation.

Table 5. Analgesic activity of 10*H*-phenothiazine-1-acylhydrazone derivatives (**6a–i**) and acylhydrazide intermediate (**12**)

Compound	W or X	*n*	Constrictions number	Inhibition (%)
Vehicle control	—	16	75.5 ± 4.1	—
Indometacin	—	10	34.1 ± 3.8	54.9*
Dipyrone	—	10	48.40 ± 6.7	35.9*
6a	W = 4-H	9	47.4 ± 8.1	37.1*
6b	W = Br	9	46.2 ± 5.2	38.8*
6c	W = 4-OCH_3	9	51.6 ± 4.2	31.7*
6d	W = NO_2	8	24.1 ± 4.8	68.0*
6e	W = 4-$N(CH_3)_2$	12	57.9 ± 6.4	23.4
6f	W = 3,5-di-tertbutyl-4-OH	10	49.1 ± 6.1	35.0*
6g	W = COOH	14	23.1 ± 4.9	69.4*
6h	X = O	8	26.9 ± 7.4	24.7*
6i	X = S	10	53.5 ± 5.9	29.1*
12	—	11	57.7 ± 5.7	23.4*

All compounds were administered *p.o.* at a dose of 100 μmols/kg. % of inhibition obtained by comparison with vehicle control group. Results are expressed as mean ± SEM. *n* = number of animals.
* $p < 0.05$ (Student's '*t*' test).

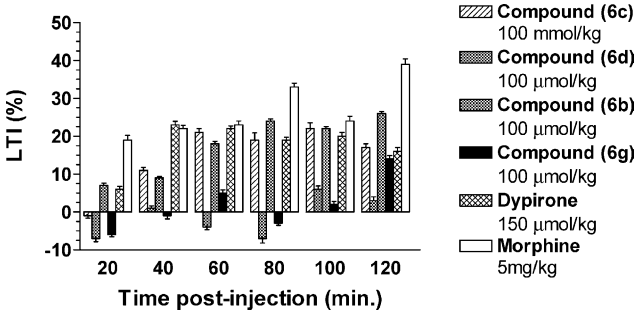


Chart 1. Effect of *p.o.* administration of 10*H*-phenothiazine-1-acylhydrazone derivatives (**6b–d**) and (**6g**) on the course of the latency times in the hot plate test in mice.

The IC_{50} values obtained for compounds (**6a**), (**6h**), and (**12**) showed that they are more potent for the inhibition of AA- than collagen-induced aggregation (Table 7). The most active compound was unsubstituted acylhydrazone derivative (**6a**) with an $IC_{50} = 2.3\ \mu M$, which was approximately twofold more potent than isosteric 2-furyl derivative (**6h**), that is $IC_{50} = 5.4\ \mu M$ and more than 10-fold more active than prototype compound (**3**). The acylhydrazide intermediate (**12**) was the less active compound, presenting an $IC_{50} = 18.4\ \mu M$ (Table 7).

AA-induced aggregation occurred essentially through the stimulation of the TXA_2 bioformation, thus the results described herein suggested that acylhydrazone derivatives (**6** and **12**) are more selective inhibitors for the AA pathway than others mechanisms involved in platelet activation.

As concluding remarks, we discovered a new potent prototype of antiplatelet derivative (**6a**), which acts in AA pathway probably by inhibition of platelet COX-1 enzyme. Additionally, the change of *para*-substituent group of acylhydrazone framework permitted us to identify hydrophilic carboxylate derivative (**6g**) and hydrophobic bromo derivative (**6b**) as two new leads of analgesics more

Table 6. Platelet anti-aggregating activity of 10*H*-phenothiazine-1-acylhydrazone derivatives (**6a–i**) and acylhydrazide intermediate (**12**)

Compound[a]	AA (200 μM)			Collagen (5 μg/mL)			ADP(5 μM)		
	N[d]	Aggregation[c] (%)	Inhibition (%)	N[d]	Aggregation[c] (slope)	Inhibition (%)	N[d]	Aggregation[c] (%)	Inhibition (%)
Control	6	89.5 ± 3.2	—	5	13.6 ± 0.6	—	8	57.2 ± 3.2	—
Indomethacin[b]	4	0.0 ± 0.0	100*	4	0.5 ± 0.3	94.8*	5	—	2.0
6a	3	0.0 ± 0.0	100*	3	0.2 ± 0.2	98.7*	3	58.9 ± 9.5	3.0
6b	4	3.5 ± 3.5	96.2*	4	2.7 ± 1.6	79.9*	4	51.0 ± 5.9	10.9
6c	4	0.0 ± 0.0	100*	4	2.5 ± 1.1	81.9*	3	51.9 ± 2.6	9.2
6d	3	0.0 ± 0.0	100*	3	0.9 ± 0.5	93.4*	3	50.7 ± 1.3	11.4
6e	4	3.7 ± 2.5	95.9*	4	0.7 ± 0.5	95.1*	3	56.5 ± 6.6	1.2
6f	3	0.0 ± 0.0	100*	3	1.4 ± 1.2	89.9*	—	54.2 ± 2.7	5.2
6g	3	0.0 ± 0.0	100*	3	0.8 ± 0.8	93.9*	3	54.9 ± 4.6	3.9
6h	3	0.0 ± 0.0	100*	4	0.8 ± 0.6	94.1*	4	52.3 ± 4.6	8.7
6i	4	0.0 ± 0.0	100*	4	0.7 ± 0.7	95.2*	3	58.0 ± 10.6	−1.3
12	3	0.0 ± 0.0	100*	3	0.8 ± 0.8	94.3*	3	52.1 ± 2.3	8.8

[a] Test compounds were orally administered at a dose of 100 μM.
[b] Used as standard at a dose of 10 μM.
[c] Results are expressed as mean ± SEM.
[d] N = number of experiments in triplicate.
* $p < 0.05$ (Student's '*t*' test).

Table 7. IC_{50} of the compounds (**6a**), (**6h**), and (**12**) on the platelet aggregation induced by collagen and AA

Compound	IC_{50} (μM)	
	Collagen (5 μg/mL)	AA (200 μM)
6a	39.3 ± 4.9	2.3 ± 0.4
6h	23.0 ± 1.4	5.4 ± 0.2
12	27.9 ± 1.4	18.4 ± 0.3

active than dipyrone used as standard and with selective peripheral or central mechanism of action.

4. Experimental protocols

4.1. Chemistry

Melting points were determined with a Quimis 340 apparatus and are uncorrected. ^{1}H NMR spectra were determined otherwise in deuterated chloroform or dimethylsulfoxide containing ca. 1% tetramethylsilane as an internal standard, with Brucker AC 200 or Varian Gemini 200 and Varian Gemini 300 at 200 and 300 MHz, respectively. ^{13}C NMR spectra were determined in the same spectrometers described above at 50 or 75 MHz, employing the same solvents. IR spectra were obtained with Jasco Valor III and Perkin Elmer 283-B spectrophotometers by using potassium bromide pellets. UV spectra were determined in methanol (TEDIA) solution on a Shimadzu UV 1601 spectrophotometer. HPLC analyses were performed on a Shimadzu CBM-10A, equipped with a Rexchrom 5 μm RP-18 column (125×4.6 mm). Analysis was done in the isocratic mode, using a mixture of methanol:water (7:3 v/v) as eluent at flow rate of 1 mL/min. Mass spectra were obtained with a Auto Specq EI at 70 eV.

The progress of all reactions was monitored by TLC, which was performed on 2.0×6.0 cm aluminum sheets precoated with silica gel 60 (HF-254, Merck) to a thickness of 0.25 mm. The developed chromatograms were viewed under ultraviolet light (254–265 nm) and treated with iodine vapor. The usual work-up means that the organic extracts prior to concentration under reduced pressure, were treated with a saturated aqueous sodium chloride solution, referred as to brine, dried over anhydrous sodium sulfate, and filtered.

4.1.1. 10*H*-Phenothiazine (8). This compound was obtained in 89% yield following the procedure previously described by Fitton et al.[11] mp 183–184 °C (lit.). IR (KBr) cm^{-1}: 3342 (*v* N–H), 3184 (*v* Ar–H), 1598–1572 (*v* C=C), 1471–1413 (*δ* C–N), 751–717 (Ar, di *1,2*);[26] ^{1}H NMR (300 MHz, $CDCl_3$): *δ* 5.8 (s, N–*H*), 6.4–7.1 (m, 8H, Ar–*H*); ^{13}C NMR (75 MHz, $CDCl_3$): *δ* 115.01 (C-1, 9), 127.92 (C-2, 8), 123.19 (C-3, 7), 127.41 (C-4, 6), 118.85 (C-4a, 6a), 142.22 (C-9a, 10a);[27] UV (nm): 437 ($n \rightarrow \pi$), 317, 254 ($\pi \rightarrow \pi$); MS (70 eV) *m/z* (relative abundance): 199 (100%), 167 (42%).

4.1.2. 1.2-Diidropyrrolo[3,2,1-kl]-phenothiazin-1,2-dione (9). This compound was obtained in 81% yield following the procedure previously described by Lopes et al.[13] mp 202–204 °C (lit.). IR (KBr) cm^{-1}: 1740 (*v* C=O, amide group), 1721 (*v* C=O, ketone group); ^{1}H NMR (300 MHz, $CDCl_3$): *δ* 8.5 (dd, *H*-9, *J* = 1.3 and 8.3 Hz), 7.2 (dd, *H*-2, *J* = 1.5 and 7.0 Hz), 6.9 (t, *H*-3, *J* = 7.5 Hz), 6.8 (dd, *H*-4, *J* = 1.6 e 7,7 Hz), 7.0 (td, *H*-8, *J* = 1.3 and 7.6 Hz), 6.7 (td, *H*-7, *J* = 13 and 7.6 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): *δ* 116.23 (C-1), 122.84 (C-2), 126.78 (C-3), 133.23 (C-4), 117.50 (C-4a), 117.40 (C-6a), 126.78 (C-6), 126.37 (C-7), 127.85 (C-8), 118.54 (C-9), 131.00 (C9a), 143.29 (C10a), 155.18 (C=O), 180.70 (C=O); UV (nm): 520, 436 ($n \rightarrow \pi$), 274 ($\pi \rightarrow \pi$); MS (70 eV) *m/z* (relative abundance): $C_{14}H_7NO_2S$, 253 (13%), 225 (50%), 197 (100%).

4.1.3. 10*H*-Phenothiazine-1-carboxylic acid (10). To a suspension of 2.2 g (8.69 mmol) of isatin derivative (**8**) in 150 mL of water maintained at 30 °C were added 30 mL of 30% aq NaOH solution. Next, the reaction mixture was stirred for 1 h at room temperature and then we added slowly 75 mL of water and 5 mL of 30% H_2O_2 diluted in 50 mL of water. After 2 h, the resulting solution was neutralized with concentrated HCl, resulting in the formation of a yellow precipitate, which was filtered, washed with water and dried under reduced pressure to give 2.55 g of the carboxylic acid derivative (**10**) (88% yield). mp 261–263 °C; IV (KBr) cm^{-1}: 3500 (*v* N–H), 3200–2500 (*v* O–H broad), 1662 (*v* C=O); ^{1}H NMR (300 MHz, DMSO-d_6): *δ* 10.0 (s, N–*H*), 3.5 (O–*H*, broad) 7.6 (dd, H-2, *J* = 1.23 and 7.9 Hz), 6.7 (t, H-3, *J* = 7.6 Hz), 7.1 (dd, H-4, *J* = 1.3 and 7.4 Hz), 6.8 (td, H-7, *J* = 1.3 and 7.4 Hz), 6.9 (dd, H-6, 1.3 e 7.4 Hz), 6.9 (td, H-8, *J* = 1.3 and 7.5 Hz), 6.6 (dd, H-9, *J* = 1.2 and 7.5 Hz); RMN ^{13}C (75 MHz, DMSO-d_6): *δ* 112.19 (C-1), 129.68 (C-2), 120.88 (C-3), 131.06 (C4), 118.39 (C-4a), 116.60 (C-6a), 126.31 (C-6), 123.31 (C-7), 127.91 (C-8), 139.73 (C-9a), 144.79 (C-10a), 169.46 (CO).[28]

4.1.4. Methyl 10*H*-phenothiazine-1-carboxylate (11). This compound was obtained in quantitative yield following the procedure previously described by Boekelheide and Hollins[12] mp 113–114 °C. IR (KBr) cm^{-1}: 3293 (*v* N–H), 1690 (*v* C=O); ^{1}H NMR (300 MHz, $CDCl_3$): *δ* 9.92 (s, N–*H*), 7.6 (dd, H-2, *J* = 1.6 and 8.2 Hz), 6.7 (t, H-3, *J* = 8.0 Hz), 7.1 (dd, H-4, *J* = 1.5 and 7.6 Hz), 6.9 (dd, H-6, *J* = 1.6 and 7.5 Hz), 6.8 (d, H-7, *J* = 1.2 and 7.3 Hz), 7.0 (td, H-8, *J* = 1.5 and 7.6 Hz), 6.6 (dd, H-9, *J* = 1.2 and 7.9 Hz), 3.9 (s, OCH_3); ^{13}C NMR (75 MHz, $CDCl_3$): *δ* 52.05 (OCH_3), 111.34 (C-1), 129.15 (C-2), 120.41 (C-3), 131.16 (C-4), 119.68 (C-4a), 117.63 (C-6a), 126.38 (C-6), 123.20 (C-7), 127.56 (C-8), 115.74 (C-9), 140.09 (C-9a), 145.67 (C-10a), 168.52 (CO); UV (nm): 399 ($n \rightarrow \pi$), 255 ($\pi \rightarrow \pi$); MS (70 eV) *m/z* (relative abundance): 257 (73%), 225 (55%), 197 (100%).

4.1.5. 10*H*-Phenothiazine-1-carbohydrazide (12). To a solution of 2.0 g (7.78 mmol) of the methyl ester derivative (**11**) and 10 mL of 55% aq hydrazine monohydrate (320 mmol) in 40 mL of absolute ethanol, was stirred at

reflux for 4 h. After concentration of the reaction mixture under reduced pressure, 30 mL of cold water was added to the residue giving the formation of a yellow precipitate, which was collected by filtration, washed with cold water and dried under vacuum to furnish 1.78 g of the desired hydrazide (**12**) (89% yield). mp 113–114 °C. IR (KBr) cm^{-1}: 3293 (*v* N–H), 1690 (*v* C=O); ^1H NMR (300 MHz, $CDCl_3$): δ 7.0 (dd, H-2, $J = 1.1$ and 9.1 Hz), 6.7 (t, H-3, $J = 7.7$ Hz), 6.9 (dd, H-4, 1.4 and 7.6 Hz), 6.9 (dd, H-6, $J = 1.5$ and 7.7 Hz), 6.8 (td, H-7, 1.2 and 7.4 Hz), 6.9 (td, H-8, $J = 1.2$ and 7.4 Hz), 6.6 (dd, H-9, $J = 7.8$ and 1.1 Hz), 7.4 (s, N–*H*), 9.8 (s, CON*H*), 4.0 (broad, NH_2); ^{13}C NMR (75 MHz, $CDCl_3$): 113.61 (C-1), 127.60 (C-2), 120.76 (C-3), 130.01 (C-4), 120.93 (C-4a), 117.44 (C-6a), 124.51 (C-6), 123.03 (C-7), 127.40 (C-8), 115.66 (C-9), 140.49 (C9a), 144.08 (C10a), 169.57 (C=O); MS (70 eV) *m/z* (relative abundance): 257 (63%), 225 (82%), 197 (100%).

4.1.6. General procedure for preparation of the 10*H*-phenothiazine-1-acylhydrazone derivatives (6a–i). To a solution of 0.150 g (0.58 mmol) of hydrazide (**12**) in absolute ethanol (15 mL) containing two drops of 37% hydrochloric acid, was added 0.60 mmol of corresponding aromatic aldehyde derivative previously diluted in absolute ethanol (5 mL). The mixture was stirred at room temperature for 30 min, until extensive precipitation was visualized. Next, the solvent was partially concentrated at reduced pressure and the resulting mixture was poured into cold water. After neutralization with 10% aqueous sodium bicarbonate solution, the precipitate formed was filtered out and dried under vacuum to give desired acylhydrazone derivatives (**6a–i**).

4.1.7. Benzylidene 10*H*-phenothiazine-1-carbohydrazide (6a). The derivative (**6a**) was obtained as a yellow solid by condensation of (**12**) with benzaldehyde (HPLC retention time = 4.54 min). ^1H NMR (200 MHz, DMSO-d_6): δ 12.01 (s, N–*H*), 8.3 (s, C–*H*) 7.8 (m, H-12), 7.7 (m, H-13), 7.4 (m, H-14); ^{13}C NMR (50 MHz, DMSO-d_6): δ 119.8 (C-1), 128.0 (C-2), 121.0 (C-3), 129.6 (C-4), 115.6 (C-4a), 116.8 (C-6a), 126.5 (C-6), 123.2 (C-7), 127.0 (C-8), 115.8 (C-9), 140.6 (C-9a), 143.4 (C-10a), 147.75 (C-11), 127.60 (C-12), 128.52 (C-13), 127.01 (C-14), 130.9 (C-15), 167.45 (C=O); IR (KBr) cm^{-1}: 3467 (*v* N–H), 1697 (*v* C=O), 1474 (δ C–O); UV (nm): 393 ($n \rightarrow \pi$), 321 ($\pi \rightarrow \pi$); MS (70 eV) *m/z* (relative abundance): 345 (70%), 225 (100%), 197 (72%).

4.1.8. (4′-Bromobenzylidene) 10*H*-phenothiazine-1-carbohydrazide (6b). The derivative (**6b**) was obtained as a yellow solid by condensation of (**12**) with 4-bromobenzaldehyde (HPLC retention time = 5.76 min). ^1H NMR (200 MHz, DMSO-d_6): δ 12.0 (s, N–*H*), 9.9 (s, N–*H*), 8.3 (s, C–*H*), 7.5 (d, H-12, $J = 7$ Hz), 6.7 (d, H-13, $J = 7$ Hz); ^{13}C NMR (50 MHz, DMSO-d_6): δ 114.5 (C-14), 115.6 (C-4a), 115.8 (C-9), 116.8 (C-6a), 119.8 (C-1), 121.0 (C-3), 123.2 (C-7), 126.5 (C-6), 126.8 (C-12), 127.0 (C-8), 128.0 (C-2), 129.0 (C-13), 129.6 (C-4), 140.6 (C-9a), 143.4 (C-10a), 147.2 (C-11), 161.1 (C-15), 164.4 (C=O); IR (KBr) cm^{-1}: 3328 (*v* N–H), 1631 (*v* C=O); UV (nm): 395 ($n \rightarrow \pi$), 315 ($\pi \rightarrow \pi$); MS (70 eV) *m/z* (relative abundance): 423 (28%), 225 (100%), 197 (80%).

4.1.9. (4′-Methoxybenzylidene) 10*H*-phenothiazine-1-carbohydrazide (6c). The derivative (**6b**) was obtained as a yellow solid by condensation of (**12**) with 4-methoxybenzaldehyde (HPLC retention time = 4.64 min). ^1H NMR (200 MHz, DMSO-d_6): δ 11.9 (s, N–*H*), 9.9 (s, N–*H*), 8.3 (s, C–*H*), 7.3 (d, H-12, $J = 7$ Hz), 7.0 (d, H-13, $J = 7$ Hz), 3.8 (s, OCH_3); ^{13}C NMR (50 MHz, DMSO-d_6): 55.4 (OCH_3), 119.8 (C-1), 128.0 (C-2), 121.0 (C-3), 129.6 (C-4), 115.6 (C-4a), 116.8 (C-6a), 126.5 (C-6), 123.2 (C-7), 127.0 (C-8), 115.8 (C-9), 140.6 (C-9a), 143.4 (C-10a), 148.7 (C-11), 126.8 (C-12), 129.0 (C-13), 114.5 (C-14), 161.1 (C15), 164.4 (C=O); IR (KBr) cm^{-1}: 3429 (*v* N–H), 1631 (*v* C=O), 1442 (*v* C–N); UV (nm): 437($n \rightarrow \pi$), 317.254 ($\pi \rightarrow \pi$); MS (70 eV) *m/z* (relative abundance): 375 (67%), 225 (100%), 197 (75%).

4.1.10. (4′-Nitrobenzylidene) 10*H*-phenothiazine-1-carbohydrazide (6d). The derivative (**6d**) was obtained as a red solid by condensation of (**12**) with 4-nitrobenzaldehyde (HPLC retention time = 4.87 min). ^1H NMR (200 MHz, DMSO-d_6): δ 12.0 (s, N–*H*), 9.9 (s, N–*H*), 8.2 (s, C–*H*), 7.5 (d, H-12, $J = 7$ Hz), 6.7 (d, H-13, $J = 7$ Hz); ^{13}C NMR (50 MHz, DMSO-d_6): δ 114.5 (C-14), 115.6 (C-4a), 115.8 (C-9), 116.8 (C-6a), 119.8 (C-1), 121.0 (C-3), 123.2 (C-7), 126.5 (C-6), 126.8 (C-12), 127.0 (C-8), 128.0 (C-2), 129.0 (C-13), 129.6 (C-4), 140.6 (C-9a), 143.4 (C-10a), 145.81 (C-11), 147.9 (C15), 164.6 (C=O); IR (KBr) cm^{-1}: 3409 (*v* N–H), 1632 (*v* C=O); UV (nm): 407 ($n \rightarrow \pi$), 329 ($\pi \rightarrow \pi$); MS (70 eV) *m/z* (relative abundance): 390 (65%), 225 (100%), 197 (80%).

4.1.11. (4′-Dimethylaminobenzylidene) 10*H*-phenothiazine-1-carbohydrazide (6e). The derivative (**6e**) was obtained as an orange solid by condensation of (**12**) with 4-dimethylaminobenzaldehyde (HPLC retention time = 4.98 min). ^1H NMR (200 MHz, DMSO-d_6): δ 11.9 (s, N–*H*), 9.9 (s, N–*H*), 8.3 (s, C–*H*), 7.5 (d, H-12, $J = 7$ Hz), 6.7 (d, H-13, $J = 7$ Hz), 3.0 (CH_3); ^{13}C NMR (50 MHz, DMSO-d_6): δ 39.8 (NCH_3), 119.8 (C-1), 128.0 (C-2), 121.0 (C-3), 129.6 (C-4), 115.6 (C-4a), 116.8 (C-6a), 126.5 (C-6), 123.2 (C-7), 127.0 (C-8), 115.8 (C-9), 140.6 (C-9a), 143.4 (C-10a), 151.5 (C-11), 126.8 (C-12), 129.0 (C-13), 114.5 (C-14), 161.1 (C15), 164.0 (CO); IR (KBr) cm^{-1}: 3448 (*v* N–H), 1631 (*v* C=O); UV (nm): 482 ($n \rightarrow \pi$), 372 and 336 ($\pi \rightarrow \pi$); MS (70 eV) *m/z* (relative abundance): 388 (96%), 225 (100%), 197 (80%).

4.1.12. (3′,5′-Ditertbutyl-4′-hydroxybenzylidene) 10*H*-phenothiazine-1-carbohydrazide (6f). The derivative (**6f**) was obtained as a green solid by condensation of (**12**) with 3,5-ditertbutyl-4-hydroxybenzaldehyde (HPLC retention time = 6.50 min). ^1H NMR (200 MHz, DMSO-d_6): δ 9.9 (s, N–*H*), 8.3 (s, C–*H*), 7.5 (d, H-12, $J = 7$ Hz), 6.7 (d, H-13, $J = 7$ Hz), 2.5 (CH_3); ^{13}C NMR (50 MHz, DMSO-d_6): δ 30.1 (CH_3), 114.5 (C-14), 115.6 (C-4a),

115.8 (C-9), 116.8 (C-6a), 119.8 (C-1), 121.0 (C-3), 123.2 (C-7), 126.5 (C-6), 126.8 (C-12), 127.0 (C-8), 128.0 (C-2), 129.0 (C-13), 129.6 (C-4), 140.6 (C-9a), 143.4 (C-10a), 149.5 (C-11), 156.4 (C-15), 164.0 (C=O); IR (KBr) cm^{-1}: 3633 (ν O–H), 3307 (ν N–H), 1630 (ν C=O); UV (nm): 390 ($n \rightarrow \pi$) 323 ($\pi \rightarrow \pi$); MS (70 eV) m/z (relative abundance): 473 (66%), 225 (100%), 197 (52%).

4.1.13. (4′-Carboxybenzylidene) 10*H*-phenothiazine-1-carbohydrazide (6g). The derivative (**6g**) was obtained as a yellow solid by condensation of (**12**) with 4-carboxybenzaldehyde (HPLC retention time = 10.01 min). ^{1}H NMR (200 MHz, DMSO-d_6): δ 13.0 (s, O–*H*), 12.0 (s, N–*H*), 9.9 (s, N–*H*), 8.3 (s, C–*H*), 8.2 (s, C–*H*), 7.5 (d, H-12, $J = 7$ Hz), 6.7 (d, H-13, $J = 7$ Hz); ^{13}C NMR (50 MHz, DMSO-d_6): δ 114.5 (C-14), 115.6 (C-4a), 115.8 (C-9), 116.8 (C-6a), 119.8 (C-1) 121.0 (C-3), 123.2 (C-7), 126.5 (C-6), 126.8 (C-12), 127.0 (C-8), 128.0 (C-2), 129.0 (C-13), 129.6 (C-4), 140.6 (C-9a), 143.4 (C-10a), 145.81 (C-11), 147.9 (C15), 164.0 (C=O); IR (KBr) cm^{-1}: 3100 (ν O–H), 1632 (ν C=O); MS (70 eV) m/z (relative abundance): 389 (13%), 225 (50%), 197 (100%).

4.1.14. (Furfurylidene) 10*H*-phenothiazine-1-carbohydrazide (6h). The derivative (**6h**) was obtained as a yellow solid by condensation of (**12**) with furfuraldehyde (HPLC retention time = 3.82 min). ^{1}H NMR (200 MHz, DMSO-d_6): δ 12.0 (s, N–H), 9.9 (s, N–*H*), 8.3 (s, C–*H*), 7.5 (d, H-12, $J = 3.3$ Hz), 7.1 (dd, H-13, $J = 3.3$ Hz), 7.7 (d, H-13′, $J = 4.2$ Hz); ^{13}C NMR (50 MHz, DMSO-d_6): δ 114.5 (C-14), 115.6 (C-4a), 116.8 (C-6a), 119.8 (C-1), 121.0 (C-3), 123.2 (C-7), 126.5 (C-6), 126.8 (C-12), 127.0 (C-8), 128.0 (C-2), 129.0 (C-13), 129.6 (C-4), 115.8 (C-9), 140.6 (C-9a), 143.4 (C-10a), 145.30 (C-11), 147.9 (C-15), 164.2 (C=O); IR (KBr) cm^{-1}: 3303 (ν N–H), 1628 (ν C=O); UV (nm): 391 ($n \rightarrow \pi$) 317 ($\pi \rightarrow \pi$); MS (70 eV) m/z (relative abundance): 335 (75%), 225 (100%), 197 (92%).

4.1.15. (Thienylidene) 10*H*-phenothiazine-1-carbohydrazide (6i). The derivative (**6i**) was obtained as a yellow solid by condensation of (**12**) with thiophene-2-carboxaldehyde (HPLC retention time = 4.10 min). ^{1}H NMR (200 MHz, DMSO-d_6): δ 12.0 (sl, N–*H*), 10.5 (s, N–*H*), 8.3 (s, C–*H*), 7.7 (d, H-13′, $J = 4.2$ Hz), 7.5 (d, H-12, $J = 3.3$ Hz), 7.1 (dd, H-13, $J = 3.3$ Hz); ^{13}C NMR (50 MHz, DMSO-d_6): δ 114.5 (C-14), 115.6 (C-4a), 115.8 (C-9), 116.8 (C-6a), 119.8 (C-1) 128.0 (C-2), 121.0 (C-3), 123.2 (C-7), 126.5 (C-6), 126.8 (C-12), 127.0 (C-8), 129.0 (C-13), 129.6 (C-4), 140.6 (C-9a), 142.9 (C-11), 143.4 (C-10a), 147.9 (C-15), 165.1 (C=O); IR (KBr) cm^{-1}: 3282 (ν N–H), 1630 (ν C=O); UV (nm): 393 ($n \rightarrow \pi$) 325 ($\pi \rightarrow \pi$); MS (70 eV) m/z (relative abundance): 351 (70%), 225 (75%), 197 (100%).

4.2. Computational chemistry

4.2.1. Molecular modeling. The geometry optimization of benzylidene 10*H*-phenothiazine-1-acylhydrazone derivative (**6a**) was performed using the semiempirical AM1 Hamiltonian[16] within SPARTAN 1.0.5 program[29] on a Pentium IV 1.5 GHz.

4.3. Pharmacology

4.3.1. Preparation of rabbit and human platelet rich plasma. Rabbit blood was collected from the central ear artery from rabbits weighing 2.5–3.0 kg. Human blood was obtained by vein puncture of the median cubital vein from healthy volunteers who had not taken any medication for at least 15 days before the study. Blood samples were collected into 3.8% trisodium citrate (9:1 v/v). Platelet-rich plasma (PRP) was prepared by centrifugation at 500 × g for 10 min at room temperature. The platelet pour plasma (PPP) was prepared by centrifugation of the pellet at 1800 × g for 10 min at room temperature. Platelet count was adjusted to 5×10^8 mL^{-1}.

4.3.2. Platelet aggregation. Platelet aggregation was monitored by the turbidimetric method of Born and Cross[23] in a Chrono-Log aggregometer. PRP (400 μL) was incubated at 37 °C for 1 min with continuous stirring at 900 rpm. Aggregation of PRP was induced by ADP (5 μM), collagen (5 μg/mL), arachidonic acid (200 μM). Test compounds and the vehicle (0.5% DMSO, 2 μL) were added to the PRP samples 5 min before addition of the aggregating agent. The DMSO used as vehicle did not have either pro- or antiplatelet aggregation activity. The platelet aggregation was expressed as percentage of aggregation for ADP, AA, and as the maximum rate of aggregation (slope) for collagen.

4.3.3. Analgesic activity. The analgesic activity was determined in vivo by the abdominal constriction test induced by acetic acid 0.6% (0.1 mL/10 g) in mice.[19] Albino mice of both sexes (18–23 g) were used. Compounds were administered orally (100 μmol/kg; 0.1 mL/20 g) as a suspension in 5% arabic gum in saline (vehicle). Indomethacin (100 μmol/kg) and dipyrone (100 μmol/kg) were used as standard drugs in the same conditions. Acetic acid solution was administered i.p. one hour later. Ten minutes following i.p. acetic acid injection the number of constrictions per animal was recorded for 20 min. Control animals received an equal volume of vehicle. Analgesic activity was expressed as % of inhibition of constrictions when compared with the vehicle control group.

4.3.4. Antiinflammatory activity. The antiinflammatory activity was determined in vivo using the carrageenan-induced rat paw edema test according to Ferreira.[18] Fasted albino rats of both sexes (150–200 g) were used. Compounds were administered orally and intraperitoneally (100 μmol/kg; 0.1 mL/20 g) as a suspension in 5% arabic gum in saline (vehicle). Control animals received an equal volume of vehicle. One hour later, the animals were then injected with either 0.1 mL of 1% carrageenan solution in saline (0.1 mg/paw) and sterile saline (NaCl

0.9%), into the subplantar surface of one of the hind paw, respectively. The paw volume were measured using a glass plethysmometer coupled to an peristaltic pompe, at each hour, until four hours after the subplantar injection. The edema was calculated as the volume variation by the volume difference between the carrageenan and saline treated paw. Indomethacin (100 μmol/kg) was used as standard drug in the same conditions. Antiinflammatory activity was expressed as % of inhibition of the edema when compared with the vehicle control group.

4.3.5. Hot plate test. The central analgesic activity was determined in vivo by the hot plate test according to Kuraishi et al.[21] Swiss mice of both sexes (18–25 g) were used, maintained with water ad libitum and fasted for 8 h. Animals were placed on a plate heated at 55 ± 0.1 °C and their responses to thermal stimulation (licking or withdraw of the hind paw) were timed. Three control measures were done (in the absence of the test drugs) in intervals of 30 min to determine the control latency mean time and the cut-off time (maximum time of permanence of the animal in the plate), calculated as three times the control mean value (25 seg). The response time for each mouse was registered at 20 min intervals after drug administration for a total of 120 min. Data are expressed as latency time index (%).

Statistics: Data was analyzed statistically by analysis of variance (one-way, Scheffé test) and by the Student's '*t*' test for a $^{*}p$ value <0.05 and were expressed as mean ± s.e.m. for *n* experiments in triplicate.

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