RING-OPENED ANALOGS OF INDOMETHACIN AFFECTING HUMAN NEUTROPHIL FUNCTIONS+

Aldo ANDREANI[a1,*], Alberto LEONI[a2], Alessandra LOCATELLI[a3], Rita MORIGI[a3], Mirella RAMBALDI[a], Jean-Claude GEHRET[b], Serena TRANIELLO[c1], Alessio CARIANI[c2] and Susanna SPISANI[c2]

[a] *Department of Pharmaceutical Sciences, University of Bologna, Via Belmeloro 6, 40126 Bologna, Italy; e-mail: [1] aldoandr@alma.unibo.it, [2] aleoni@alma.unibo.it, [3] aleloc@alma.unibo.it*

[b] *Novartis Crop Protection AG, P.O. Box, CH-4002 Basel, Switzerland; e-mail: jc.gehret@cp.Novartis.com*

[c] *Department of Biochemistry and Molecular Biology, University of Ferrara, Via L. Borsari 46, 44100 Ferrara, Italy; e-mail: [1] trs@unife.it, [2] sps@unife.it*

Received July 27, 1998
Accepted January 8, 1999

Dedicated to the memory of Miroslav Protiva, professor at the University of Bologna in 1988.

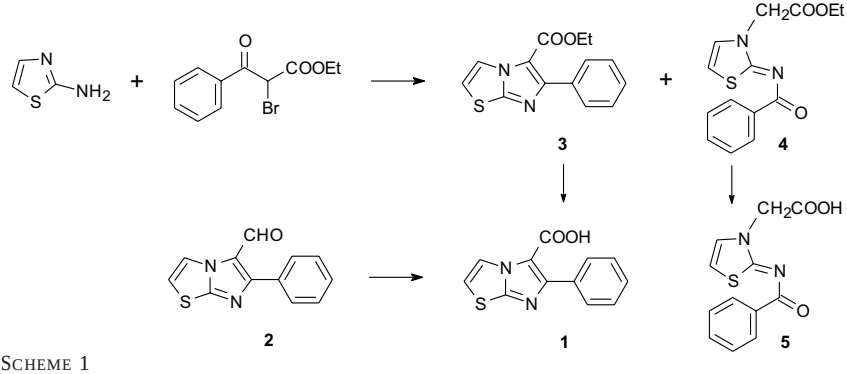
A series of ring-opened analogs of indomethacin was synthesized and tested *in vitro* (at concentrations ranging from 10^{-9} to 10^{-5} mol/l) on human neutrophil functions. Two compounds lacking the carboxylic group were subjected to the same tests and one of these showed unexpected activity. Among the acidic derivatives, compound **12** bearing the same substituents as indomethacin **10** (methoxy and 4-chlorobenzoyl groups) was the most active: it significantly lowered neutrophil responses in all five bioassays and at the three concentrations considered.

Key words: Imidazo[2,1-*b*]thiazoles; Thiazoles; Indomethacin; Inflammation; Neutrophil functions; Antiinflammatory agents.

While we were involved[1] in a project on the synthesis of antiinflammatory agents derived from 6-phenylimidazo[2,1-*b*]thiazole-5-carboxylic acid (**1**) (Scheme 1), we decided to evaluate two routes for the synthesis of this starting material: the oxidation of the corresponding aldehyde **2**, according to the method we previously described[2] and the hydrolysis of the corresponding ester **3**, reported by Tedeschi[3]. When we repeated this synthesis of **3**

+ Presented at the *First Italian-Swiss Meeting on Medicinal Chemistry (Torino, Italy, September 23–26, 1997).*

from 2-aminothiazole and ethyl 2-benzoyl-2-bromoacetate, we obtained the expected compound **3** and a by-product which, from the first spectroscopic data, was considered to be 2-benzoyliminothiazole-3-acetate **4**. The compound was then hydrolyzed and the resulting carboxylic acid **5** was subjected to a series of agrochemical tests. The level of herbicidal activity was sufficient to consider compound **5** as a lead for the synthesis of new analogs.

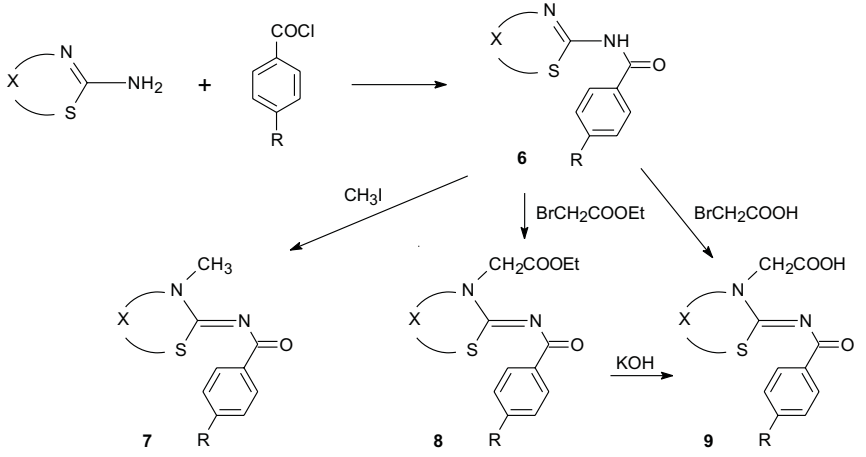


SCHEME 1

While we were involved in this project and were verifying the structure of compounds **4** and **5** by their independent synthesis, a spectroscopic demonstration of the structure of compound **4** and a plausible mechanism of its formation was suggested by Abignente *et al.*[4]. These data are in agreement with the results obtained from our independent synthesis which, in addition, was employed for the preparation of a series of analogs **6**–**9** (Scheme 2) in order to evaluate the effect of the performed structural changes on the herbicidal activity. The changes included several substituents R on the phenyl ring in esters **8** and acids **9**, and also corresponding *N*-methyl derivatives **7** were prepared.

All the changes were performed for the parent thiazole compound as well as its 4,5-dihydro derivative. However, the data obtained from the herbicidal screening did not disclose any derivative more active than **5**. Since the structure of compounds **9** is related to that of indomethacin **10** with the five-membered ring opened, we thought to test a number of these compounds as antiinflammatory agents. The activity of some opened analogues has already been successfully explored, for example, with the synthesis of clamidoxic acid[5] **11**. With this in mind, we used the same methodology for preparation of a more similar analog, compound **12**. We subjected to bio-

logical tests all the prepared acids and, since the antiinflammatory activity was not expected within this class of compounds, only two selected *N*-methyl derivatives.

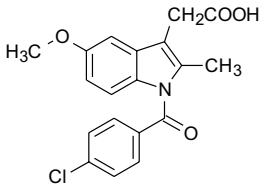


6-9	X	R
a	CH=CH	H
b	CH=CH	Cl
c	CH=CH	CH_3
d	CH=CH	NO_2
e	CH_2-CH_2	H
f	CH_2-CH_2	Cl
g	CH_2-CH_2	CH_3
h	CH_2-CH_2	NO_2

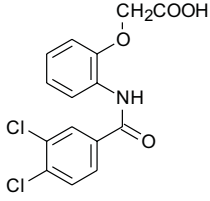
SCHEME 2

The tests we decided to employ, evaluate the effects that a given compound induces on human neutrophil functions: the activity was compared to that of indomethacin as the reference drug. It is well known that human neutrophils play central roles in both host defenses against microorganisms and tissue destruction associated with many inflammatory processes. A number of highly specialized functions allow these cells to accomplish this complicated task and it seems likely that nonsteroidal antiinflammatory drugs (NSAIDs) may, at least in part, affect the inflammatory response through their action on these cells, independent of cyclooxygenase inhibition[6]. It has been demonstrated that antiinflammatory agents inhibits several neutrophil functions involved in the development of inflammatory response such as locomotion and intracellular killing of ingested microorganisms through oxygen dependent and/or independent mechanisms[7]. The pattern of inhibition varied from drug to drug and was related to the differ-

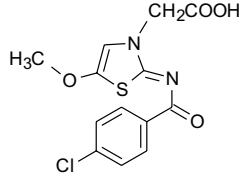
ent stimulating agents[8]. In the present study, the effect of compounds **7b**, **7f**, **9a**–**9h** and **12** was examined on *in vitro* neutrophil activation considering several aspects of human neutrophil antimicrobial functions, namely motility induced by chemoattractants, superoxide anion (O_2^-) generation and lysozyme degranulation triggered by agonists. These functions were evaluated after cell pulse with different concentrations of the drugs.



10



11



12

RESULTS AND DISCUSSION

Chemistry

For the synthesis of the 2-(benzoylamino)thiazoles **6** (Scheme 2), 2-aminothiazole or its 4,5-dihydro derivative was treated with the appropriate benzoyl chloride in the presence of triethylamine. When the reaction mixture was left at room temperature for a few hours, some dibenzoyl derivative was also obtained. However, when the reaction was performed at lower temperature, the desired monosubstituted derivatives **6** were obtained as the only isolated products in good yields. All these compounds, with the exception of compound **6c**, have already been reported in the literature[9–13]. Compounds **6** treated with iodomethane in the presence of potassium carbonate in acetone reacted as their imino forms to provide corresponding *N*-methyl derivatives **7**. Analogous treatment of sodium salts of compounds **6**, generated *in situ* with sodium hydride in DMF, with ethyl bromoacetate afforded ethyl 2-(benzoylimino)thiazole-3-acetates **8**. Carboxylic acids **9** and **12** were prepared similarly using directly bromoacetic acid in aqueous ethanolic solution of sodium hydroxide. These carboxylic acids could also be prepared by hydrolysis of the corresponding esters **8** but the yields are lower.

The spectroscopic data of the new compounds are in agreement with the assigned structures. In the IR spectra of benzoyl derivatives **6** it is possible to see the stretching vibrations of the carbonyl group at about 1 660 cm^{-1}

in the thiazole series and at about 1 610 cm^{-1} in the dihydrothiazole series. These two series are quite different even in the region of the NH group: a low intensity band is present in thiazoles (3 140 cm^{-1}) whereas a much stronger band characterizes the corresponding dihydro derivatives (3 200 cm^{-1}). The ^1H NMR spectra of thiazole derivatives show typical aromatic behaviour with two doublets in the range of 7–7.6 ppm, whereas their dihydro analogs show usual aliphatic pattern with two triplets in the region of 3.2–4.2 ppm. Under the experimental conditions employed, the resonance of the carboxylic group (compounds **9a**–**9h** and **12**) was usually undetectable.

Biological Activity

In the first step of our study we verified if the 2-(benzoylimino)thiazoles and indomethacin were chemotaxins for human neutrophils, secretagogue agents or were able per se to trigger superoxide anion production. None of the molecules was able to mediate these activities (data not shown). In the second step we tested the possible influence of the compounds on neutrophil functions.

In order to study the initial event of inflammation, we analyzed *in vitro* the influence of the tested compounds, including indomethacin (**10**) on random locomotion. All compounds showed slight effects: however these were not statistically significant ($p > 0.05$) at any of the concentrations tested (10^{-9}, 10^{-7} and 10^{-5} mol/l). Conversely, when the same compounds were tested for their ability to modulate directed migration (Table I), compounds **9b** and **12** caused a 55% inhibition ($p < 0.01$) on chemotaxis induced by FMLP (*N*-formylmethionylleucylphenylalanine) at a concentration as low as 10^{-9} mol/l, comparable to the effect of indomethacin at 10^{-5} mol/l. Compounds **9c**, **9d** and **12** behaved like the reference drug at 10^{-5} mol/l. A slight but significant ($p < 0.05$) inhibition of chemotaxis was observed after cell treatment with **7b**, **7f**, **9a** and **9e**. Other tested compounds proved to be inactive in this function. When chemotaxis was induced by casein (2 mg/ml), compounds **9b**, **9g**, **9h** and **12** displayed 35–40% ($p < 0.05$) inhibitory effect at the lowest concentration.

The modulation of the selected compounds and indomethacin on FMLP-induced respiratory burst is shown in Table I. Only compounds **7f**, **9b**, **9f** and **12** were able to induce a statistically significant ($p < 0.05$) inhibition on free radical production: the greatest activity was at 10^{-5} mol/l for compounds **9b**, **12** and the reference drug and at 10^{-9} mol/l for compounds **7f** and **9f**. The other analogs gave no effect. Moreover, no effect was seen

TABLE I

Activity of compounds **7b**, **7f**, **9a**–**9h** and **12** on human neutrophil functions (in %) in comparison with indomethacin **10** (only the significant values are reported[a])

Compound	Concentration mol/l	FMLP-induced chemotaxis	Casein-induced chemotaxis	FMLP-triggered O_2^- generation	FMLP-induced lysozyme secretion	PMA-induced lysozyme secretion
10	10^{-9}	55	85	78	78	65
	10^{-7}	48	67	78	76	64
	10^{-5}	41	66	64	65	59
7b	10^{-9}	77				73
	10^{-7}	68				65
	10^{-5}	63				64
7f	10^{-9}	78		71	75	67
	10^{-7}	58		79	69	67
	10^{-5}	56		77	69	53
9a	10^{-9}	55	75			
	10^{-7}	57	68			
	10^{-5}	55	68			
9b	10^{-9}	46	64	80		
	10^{-7}	46	67	80		
	10^{-5}	41	71	70		
9c	10^{-9}	85	86		70	63
	10^{-7}	65	80		75	64
	10^{-5}	39	70		72	69
9d	10^{-9}	62			69	64
	10^{-7}	62			68	64
	10^{-5}	51			70	59
9e	10^{-9}	84	73		72	82
	10^{-7}	68	70		72	72
	10^{-5}	67	55		71	62
9f				75		
				83		
				84		
9g	10^{-9}		58		77	71
	10^{-7}		70		76	70
	10^{-5}		76		75	68
9h	10^{-9}		58		78	68
	10^{-7}		59		78	70
	10^{-5}		63		75	69
12	10^{-9}	45	65	89	71	51
	10^{-7}	44	65	82	70	55
	10^{-5}	41	62	78	69	54

[a] Each value is the average ± SEM from 8–10 separate experiments carried out in triplicate and SEM are around 10%. Statistical significance is reported in the text.

for all tested compounds when respiratory burst was triggered by PMA (phorbol 12-myristate 13-acetate). These data are in agreement with previous reports[8,15,16] where the inhibitory action of NSAIDs was predominantly on responses mediated through the FMLP receptor, probably by disrupting molecular interactions of key components of the transduction machinery within the plasma membrane[17].

When the derivatives were tested for their ability to influence FMLP- or PMA-dependent lysozyme release, about 35% dose independent-inhibition (very close to that of indomethacin) was seen for several derivatives (Table I). Compound **12** showed a high inhibition ($p < 0.01$) at the lowest concentration when the agonist was PMA.

The aim of this study was the search for new compounds which could be able to act at low concentrations, with the capability to depress neutrophil responses to inflammatory stimuli thus reducing unwanted tissue damage. The unexpected broad activity of compound **7f** may be considered as a helpful suggestion for our next projects. As far as the carboxylic acids are concerned, the biochemical behaviour of compound **9b** is noteworthy since a significant depression of neutrophil chemotaxis and O_2^- production were found. Compound **12**, structurally the closest to indomethacin, was the most active derivative. It was capable to inhibit all aspects of neutrophil functions reported here: locomotion, release of oxygen radical and lysozyme secreted by activated neutrophils which may be important causes of tissue damage in a number of inflammatory conditions[18]. It is interesting to observe that both **9b** and **12** were able to depress directed migration towards the two chemotaxins tested, at a concentration as low as 10^{-9} mol/l.

EXPERIMENTAL

Chemistry

Melting points are uncorrected. IR spectra were taken on a Perkin–Elmer 683 spectrophotometer (wavenumbers in cm^{-1}). ^1H NMR spectra were recorded on a Varian Gemini instrument (300 MHz) in DMSO-d_6 using TMS as the internal standard. Chemical shifts are given in ppm (δ-scale), coupling constants (*J*) in Hz. Thin layer chromatography was done on pre-coated TLC plates Bakerflex (silica gel IBF-2) using mixtures of petroleum ether and acetone as eluents.

2-(Benzoylamino)thiazoles **6a**–**6h** and 2-(4-Chlorobenzoylamino)-5-methoxythiazole. General Procedure

Triethylamine (3 g, 30 mmol) was added to a solution of 30 mmol of 2-aminothiazole (preparation of compounds **6a**–**6d**) or 2-amino-4,5-dihydrothiazole (preparation of compounds **6e**–**6h**) or 2-amino-5-methoxythiazole[14] (preparation of 2-(4-chlorobenzoylamino)-

5-methoxythiazole) in tetrahydrofuran (70 ml) and the mixture was cooled to –5 °C. A solution of the appropriate benzoyl chloride (28 mmol) in tetrahydrofuran (20 ml) was then added dropwise while the temperature of the mixture was kept between –5 and 0 °C. The formed precipitate of triethylamine hydrochloride was filtered off, the filtrate was evaporated under reduced pressure and the crude product was recrystallized from ethanol to provide compounds **6a**–**6h**.

2-(Benzoylamino)thiazole (**6a**). Yield 85%, m.p. 150–152 °C (ref.[9], m.p. 150–151 °C).

2-(4-Chlorobenzoylamino)thiazole (**6b**). Yield 82%, m.p. 211–214 °C (ref.[10], m.p. 212.5–213 °C).

2-(4-Methylbenzoylamino)thiazole (**6c**). Yield 80%, m.p. 211–214 °C. For $C_{11}H_{10}N_2OS$ (218.3) calculated: 60.53% C, 4.62% H, 12.83% N; found: 60.57% C, 4.93% H, 13.05% N. IR: 1 300, 1 540, 1 610, 1 660. ^{1}H NMR: 2.38 s, 3 H (CH_3); 7.27 d, 1 H, *J* = 4.5 (H-4 or H-5); 7.34 d, 2 H, *J* = 9 (arom. H); 7.55 d, 1 H, *J* = 4.5 (H-4 or H-5); 7.99 d, 2 H, *J* = 9 (arom. H); 12.55 s, 1 H (NH).

2-(4-Nitrobenzoylamino)thiazole (**6d**). Yield 81%, m.p. 292–296 °C (ref.[11], m.p. 297–298 °C).

2-(Benzoylamino)-4,5-dihydrothiazole (**6e**). Yield 84%, m.p. 168–170 °C (ref.[12], m.p. 168 °C).

2-(4-Chlorobenzoylamino)-4,5-dihydrothiazole (**6f**). Yield 82%, m.p. 223–225 °C (ref.[13], m.p. 220–221 °C).

2-(4-Methylbenzoylamino)-4,5-dihydrothiazole (**6g**). Yield 82%, m.p. 224–226 °C (ref.[13], m.p. 225 °C).

2-(4-Nitrobenzoylamino)-4,5-dihydrothiazole (**6h**). Yield 80%, m.p. 275–279 °C (ref.[13], m.p. 261–262 °C).

2-(4-Chlorobenzoylamino)-5-methoxythiazole. Yield 81%, m.p. 203–207 °C. For $C_{11}H_9ClN_2O_2S$ (268.7) calculated: 49.17% C, 3.38% H, 10.42% N; found: 48.96% C, 3.49% H, 10.24% N. IR: 750, 1 310, 1 590, 1 655. ^{1}H NMR: 3.88 s, 3 H (CH_3O); 6.94 s, 1 H (H-4); 7.60 d, 2 H, *J* = 8.5 (arom. H); 8.06 d, 2 H, *J* = 8.5 (arom. H); 12.45 s, 1 H (NH).

2-(Benzoylimino)-3-methyl-2,3-dihydrothiazoles **7a**–**7h**. General Procedure

A mixture of appropriate compound **6** (5 mmol), anhydrous potassium carbonate (1.4 g, 10 mmol), iodomethane (7 g, 50 mmol) and acetone (100 ml) was refluxed for 12 h. The residue after evaporation of the reaction mixture was triturated with water, the insoluble portion was filtered off and recrystallized from ethanol to give compounds **7a**–**7h**.

2-(Benzoylimino)-3-methyl-2,3-dihydrothiazole (**7a**). Yield 90%, m.p. 112–116 °C. For $C_{11}H_{10}N_2OS$ (218.3) calculated: 60.53% C, 4.62% H, 12.83% N; found: 60.81% C, 4.44% H, 12.59% N. IR: 710, 1 480, 1 545, 1 590. ^{1}H NMR: 3.81 s, 3 H (CH_3N); 7.04 d, 1 H, *J* = 4 (H-4 or H-5); 7.50 m, 3 H (arom. H); 7.55 d, 1 H, *J* = 4 (H-4 or H-5); 8.23 m, 2 H (arom. H).

2-(4-Chlorobenzoylimino)-3-methyl-2,3-dihydrothiazole (**7b**). Yield 86%, m.p. 165–168 °C. For $C_{11}H_9ClN_2OS$ (252.7) calculated: 52.28% C, 3.59% H, 11.08% N; found: 51.97% C, 3.68% H, 10.88% N. IR: 970, 1 490, 1 550, 1 595. ^{1}H NMR: 3.82 s, 3 H (CH_3N); 7.08 d, 1 H, *J* = 4.5 (H-4 or H-5); 7.53 d, 2 H, *J* = 8.5 (arom. H); 7.58 d, 1 H, *J* = 4.5 (H-4 or H-5); 8.22 d, 2 H, *J* = 8.5 (arom. H).

3-Methyl-2-(4-methylbenzoylimino)-2,3-dihydrothiazole (**7c**). Yield 87%, m.p. 143–147 °C. For $C_{12}H_{12}N_2OS$ (232.3) calculated: 62.04% C, 5.21% H, 12.06% N; found: 61.86% C, 4.92% H, 11.92% N. IR: 760, 1 170, 1 550, 1 590. ^{1}H NMR: 2.36 s, 3 H (CH_3); 3.79 s, 3 H (CH_3N); 7.01 d, 1 H, *J* = 4.5 (H-4 or H-5); 7.27 d, 2 H, *J* = 8.5 (arom. H); 7.53 d, 1 H, *J* = 4.5 (H-4 or H-5); 8.10 d, 2 H, *J* = 8.5 (arom. H).

3-Methyl-2-(4-nitrobenzoylimino)-2,3-dihydrothiazole (**7d**). Yield 86%, m.p. 231–233 °C. For $C_{11}H_9N_3O_3S$ (263.3) calculated: 50.18% C, 3.45% H, 15.96% N; found: 50.24% C, 3.67% H, 16.03% N. IR: 845, 1 335, 1 490, 1 575. ^{1}H NMR: 3.86 s, 3 H (CH_3N); 7.16 d, 1 H, *J* = 4.5 (H-4 or H-5); 7.65 d, 1 H, *J* = 4.5 (H-4 or H-5); 8.32 d, 2 H, *J* = 9 (arom. H); 8.43 d, 2 H, *J* = 9 (arom. H).

2-(Benzoylimino)-3-methylthiazoline (**7e**). Yield 85%, m.p. 101–105 °C. For $C_{11}H_{12}N_2OS$ (220.3) calculated: 59.97% C, 5.49% H, 12.72% N; found: 60.07% C, 5.49% H, 12.72% N. IR: 710, 950, 1 550, 1 605. ^{1}H NMR: 3.17 t, 2 H, *J* = 7.5 (H-4 or H-5); 3.21 s, 3 H (CH_3N); 3.72 t, 2 H, *J* = 7.5 (H-4 or H-5); 7.50 m, 3 H (arom. H); 8.14 m, 2 H (arom. H).

2-(4-Chlorobenzoylimino)-3-methylthiazoline (**7f**). Yield 85%, m.p. 161–165 °C. For $C_{11}H_{11}ClN_2OS$ (254.7) calculated: 51.87% C, 4.35% H, 11.00% N; found: 52.02% C, 4.58% H, 10.87% N. IR: 950, 1 085, 1 545, 1 610. ^{1}H NMR: 3.19 t, 2 H, *J* = 7.5 (H-4 or H-5); 3.22 s, 3 H (CH_3N); 3.74 t, 2 H, *J* = 7.5 (H-4 or H-5); 7.52 d, 2 H, *J* = 8.5 (arom. H); 8.14 d, 2 H, *J* = 8.5 (arom. H).

3-Methyl-2-(4-methylbenzoylimino)thiazoline (**7g**). Yield 86%, m.p. 176–179 °C. For $C_{12}H_{14}N_2OS$ (234.3) calculated: 61.51% C, 6.02% H, 11.95% N; found: 61.61% C, 5.86% H, 12.08% N. IR: 1 165, 1 260, 1 550, 1 600. ^{1}H NMR: 2.35 s, 3 H (CH_3); 3.16 t, 2 H, *J* = 8 (H-4 or H-5); 3.20 s, 3 H (CH_3N); 3.70 t, 2 H, *J* = 8 (H-4 or H-5); 7.25 d, 2 H, *J* = 8 (arom. H); 8.03 d, 2 H, *J* = 8 (arom. H).

3-Methyl-2-(4-nitrobenzoylimino)thiazoline (**7h**). Yield 88%, m.p. 222–226 °C. For $C_{11}H_{11}N_3O_3S$ (265.3) calculated: 49.80% C, 4.18% H, 15.84% N; found: 50.03% C, 4.22% H, 16.00% N. IR: 1 520, 1 560, 1 585, 1 615. ^{1}H NMR: 3.24 t, 2 H, *J* = 8 (H-4 or H-5); 3.27 s, 3 H (CH_3N); 3.78 t, 2 H, *J* = 8 (H-4 or H-5); 8.29 d, 2 H, *J* = 9 (arom. H); 8.35 d, 2 H, *J* = 9 (arom. H).

Ethyl 2-(Benzoylimino)-2,3-dihydrothiazole-3-acetates **8a**–**8h**. General Procedure

A solution of appropriate compound **6** (4 mmol) in *N*,*N*-dimethylformamide (30 ml) was treated portionwise with sodium hydride (0.3 g, 7 mmol, 50% dispersion in mineral oil). After 15 min of stirring at 25–30 °C, the mixture was cooled to 5 °C and treated with ethyl bromoacetate (1.7 g, 10 mmol) and the mixture was stirred at 90 °C for 4 h. Then the mixture was evaporated under reduced pressure, triturated with water and the insoluble portion was filtered off and crystallized from ethanol (compound **8a** from petroleum ether) to give compounds **8a**–**8h**.

Ethyl 2-(benzoylimino)-2,3-dihydrothiazole-3-acetate (**8a**). Yield 90%, m.p. 99–102 °C. (ref.[4], m.p. 100–102 °C).

Ethyl 2-(4-chlorobenzoylimino)-2,3-dihydrothiazole-3-acetate (**8b**). Yield 86%, m.p. 110–112 °C. For $C_{14}H_{13}ClN_2O_3S$ (324.8) calculated: 51.77% C, 4.03% H, 8.62% N; found: 51.58% C, 3.95% H, 8.62% N. IR: 1 010, 1 220, 1 595, 1 740. ^{1}H NMR: 1.20 t, 3 H, *J* = 7 ($C\mathbf{H}_3CH_2$); 4.18 q, 2 H, *J* = 7 ($CH_3C\mathbf{H}_2$); 5.12 s, 2 H (CH_2N); 7.09 d, 1 H, *J* = 4.5 (H-4 or H-5); 7.53 d, 2 H, *J* = 8 (arom. H); 7.59 d, 1 H, *J* = 4.5 (H-4 or H-5); 8.13 d, 2 H, *J* = 8 (arom. H).

Ethyl 2-(4-methylbenzoylimino)-2,3-dihydrothiazole-3-acetate (**8c**). Yield 85%, m.p. 106–107 °C. For $C_{15}H_{16}N_2O_3S$ (304.4) calculated: 59.19% C, 5.30% H, 9.20% N; found: 58.97% C, 5.59% H, 9.24% N. IR: 1 020, 1 210, 1 605, 1 740. ^{1}H NMR: 1.23 t, 3 H, *J* = 7 ($C\mathbf{H}_3CH_2$); 2.37 s, 3 H (CH_3); 4.20 q, 2 H, *J* = 7 ($CH_3C\mathbf{H}_2$); 5.11 s, 2 H (CH_2N); 7.06 d, 1 H, *J* = 4.5 (H-4 or H-5); 7.28 d, 2 H, *J* = 8 (arom. H); 7.57 d, 1 H, *J* = 4.5 (H-4 or H-5); 8.05 d, 2 H, *J* = 8 (arom. H).

Ethyl 2-(4-nitrobenzoylimino)-2,3-dihydrothiazole-3-acetate (**8d**). Yield 80%, m.p. 161–165 °C. For $C_{14}H_{13}N_3O_5S$ (335.3) calculated: 50.14% C, 3.91% H, 12.53% N; found: 49.99% C,

4.06% H, 12.89% N. IR: 1 220, 1 520, 1 580, 1 745. ^{1}H NMR: 1.23 t, 3 H, *J* = 7 (C**H**$_3$CH$_2$); 4.20 q, 2 H, *J* = 7 (CH$_3$C**H**$_2$); 5.19 s, 2 H (CH$_2$N); 7.18 d, 1 H, *J* = 4.5 (H-4 or H-5); 7.65 d, 1 H, *J* = 4.5 (H-4 or H-5); 8.34 m, 4 H (arom. H).

Ethyl 2-(benzoylimino)thiazoline-3-acetate (**8e**). Yield 88%, m.p. 118–121 °C. For $C_{14}H_{16}N_2O_3S$ (292.4) calculated: 57.52% C, 5.52% H, 9.58% N; found: 57.58% C, 5.31% H, 9.83% N. IR: 1 200, 1 525, 1 615, 1 730. ^{1}H NMR: 1.19 t, 3 H, *J* = 7 (C**H**$_3$CH$_2$); 3.25 t, 2 H, *J* = 8 (H-4 or H-5); 3.81 t, 2 H, *J* = 8 (H-4 or H-5); 4.14 q, 2 H, *J* = 7 (CH$_3$C**H**$_2$); 4.53 s, 2 H (CH$_2$N); 7.45 t, 2 H, *J* = 7(arom. H); 7.54 t, 1 H, *J* = 7 (arom. H); 8.09 d, 2 H, *J* = 7 (arom. H).

Ethyl 2-(4-chlorobenzoylimino)thiazoline-3-acetate (**8f**). Yield 87%, m.p. 108–111 °C. For $C_{14}H_{15}ClN_2O_3S$ (326.8) calculated: 51.45% C, 4.63% H, 8.57% N; found: 51.72% C, 4.38% H, 8.65% N. IR: 1 275, 1 525, 1 615, 1 730. ^{1}H NMR: 1.19 t, 3 H, *J* = 7 (C**H**$_3$CH$_2$); 3.27 t, 2 H, *J* = 8 (H-4 or H-5); 3.81 t, 2 H, *J* = 8 (H-4 or H-5); 4.14 q, 2 H, *J* = 7 (CH$_3$C**H**$_2$); 4.53 s, 2 H (CH$_2$N); 7.52 d, 2 H, *J* = 8 (arom. H); 8.08 d, 2 H, *J* = 8 (arom. H).

Ethyl 2-(4-methylbenzoylimino)thiazoline-3-acetate (**8g**). Yield 82%, m.p. 125–128 °C. For $C_{15}H_{18}N_2O_3S$ (306.4) calculated: 58.80% C, 5.92% H, 9.14% N; found: 59.11% C, 6.16% H, 9.29% N. IR: 1 275, 1 525, 1 615, 1 730. ^{1}H NMR: 1.20 t, 3 H, *J* = 7 (C**H**$_3$CH$_2$); 2.35 s, 3 H (CH$_3$); 3.24 t, 2 H, *J* = 8 (H-4 or H-5); 3.80 t, 2 H, *J* = 8 (H-4 or H-5); 4.14 q, 2 H, *J* = 7 (CH$_3$C**H**$_2$); 4.51 s, 2 H (CH$_2$N); 7.25 d, 2 H, *J* = 8 (arom. H); 7.98 d, 2 H, *J* = 8 (arom. H).

Ethyl 2-(4-nitrobenzoylimino)thiazoline-3-acetate (**8h**). Yield 82%, m.p. 129–132 °C. For $C_{14}H_{15}N_3O_5S$ (337.4) calculated: 49.84% C, 4.48% H, 12.46% N; found: 50.12% C, 4.56% H, 12.21% N. IR: 1 525, 1 590, 1 620, 1 725. ^{1}H NMR: 1.20 t, 3 H, *J* = 7 (C**H**$_3$CH$_2$); 3.31 t, 2 H, *J* = 8 (H-4 or H-5); 3.85 t, 2 H, *J* = 8 (H-4 or H-5); 4.16 q, 2 H, *J* = 7 (CH$_3$C**H**$_2$); 4.58 s, 2 H (CH$_2$N); 8.30 m, 4 H (arom. H).

2-(Benzoylimino)-2,3-dihydrothiazole-3-acetic Acids **9a**–**9h** and **12**. General Procedure

A solution of 2-(benzoylamino)thiazole **6a**–**6h** or 2-(4-chlorobenzoylamino)-5-methoxythiazole (4 mmol) in ethanol (150 ml) was treated with bromoacetic acid (1 g, 7 mmol) and a 2 M solution of sodium hydroxide was added dropwise to reach pH 8. The mixture was then refluxed for 16 h, evaporated under reduced pressure, the residue was treated with water and acidified with 2 M solution of hydrochloric acid. The insoluble portion was filtered off and crystallized from ethanol to give compounds **9a**–**9h** and **12**.

2-(Benzoylimino)-2,3-dihydrothiazole-3-acetic acid (**9a**). Yield 70%, m.p. 225–227 °C. For $C_{12}H_{10}N_2O_3S$ (262.3) calculated: 54.95% C, 3.84% H, 10.68% N; found: 55.27% C, 4.03% H, 10.79% N. IR: 710, 1 530, 1 590, 1 730. ^{1}H NMR: 5.08 s, 2 H (CH$_2$N); 7.05 d, 1H, *J* = 4.50 (H-4 or H-5); 7.50 m, 3 H (arom. H); 7.58 d, 1 H, *J* = 4.5 (H-4 or H-5); 8.20 m, 2 H (arom. H).

2-(4-Chlorobenzoylimino)-2,3-dihydrothiazole-3-acetic acid (**9b**). Yield 68%, m.p. 224–227 °C. For $C_{12}H_9ClN_2O_3S$ (296.7) calculated: 48.57% C, 3.06% H, 9.44% N; found: 48.29% C, 2.95% H, 9.68% N. IR: 760, 1 525, 1 580, 1 730. ^{1}H NMR: 5.06 s, 2 H (CH$_2$N); 7.07 d, 1 H, *J* = 4.5 (H-4 or H-5); 7.54 d, 2 H, *J* = 8.5 (arom. H); 7.59 d, 1 H, *J* = 4.5 (H-4 or H-5); 8.16 d, 2 H, *J* = 8.5 (arom. H).

2-(4-Methylbenzoylimino)-2,3-dihydrothiazole-3-acetic acid (**9c**). Yield 67%, m.p. 242–247 °C. For $C_{13}H_{12}N_2O_3S$ (276.3) calculated: 56.51% C, 4.38% H, 10.14% N; found: 56.75% C, 4.50% H, 9.99% N. IR: 750, 1 200, 1 530, 1 720. ^{1}H NMR: 2.37 s, 3 H (CH$_3$); 5.04 s, 2 H (CH$_2$N); 7.03 d, 1 H, *J* = 4.5 (H-4 or H-5); 7.27 d, 2 H, *J* = 8 (arom. H); 7.56 d, 1 H, *J* = 4.5 (H-4 or H-5); 8.06 d, 2 H, *J* = 8 (arom. H).

2-(4-Nitrobenzoylimino)-2,3-dihydrothiazole-3-acetic acid (**9d**). Yield 65%, m.p. 218–222 °C. For $C_{12}H_9N_3O_5S$ (307.3) calculated: 46.90% C, 2.95% H, 13.67% N; found: 47.13% C, 3.03% H, 13.58% N. IR: 1 220, 1 510, 1 540, 1 730. ^{1}H NMR: 5.11 s, 2 H (CH_2N); 7.15 d, 1 H, *J* = 4.5 (H-4 or H-5); 7.63 d, 1 H, *J* = 4.5 (H-4 or H-5); 8.31 d, 2 H, *J* = 8 (arom. H); 8.37 d, 2 H, *J* = 8 (arom. H).

2-(Benzoylimino)thiazoline-3-acetic acid (**9e**). Yield 68%, m.p. 181–183 °C. For $C_{12}H_{12}N_2O_3S$ (264.3) calculated: 54.53% C, 4.58% H, 10.60% N; found: 54.61% C, 4.86% H, 10.72% N. IR: 1 175, 1 510, 1 595, 1 730. ^{1}H NMR: 3.23 t, 2 H, *J* = 8 (H-4 or H-5); 3.80 t, 2 H, *J* = 8 (H-4 or H-5); 4.46 s, 2 H (CH_2N); 7.45 t, 2 H, *J* = 8 (arom. H); 7.53 t, 1 H, *J* = 8 (arom. H); 8.10 d, 2 H, *J* = 8 (arom. H).

2-(4-Chlorobenzoylimino)thiazoline-3-acetic acid (**9f**). Yield 65%, m.p. 187–191 °C. For $C_{12}H_{11}ClN_2O_3S$ (298.7) calculated: 48.25% C, 3.71% H, 9.38% N; found: 47.98% C, 3.63% H, 9.57% N. IR: 1 520, 1 555, 1 590, 1 720. ^{1}H NMR: 3.25 t, 2 H, *J* = 8 (H-4 or H-5); 3.81 t, 2 H, *J* = 8 (H-4 or H-5); 4.47 s, 2 H (CH_2N); 7.52 d, 2 H, *J* = 8 (arom. H); 8.10 d, 2 H, *J* = 8 (arom. H).

2-(4-Methylbenzoylimino)thiazoline-3-acetic acid (**9g**). Yield 66%, m.p. 199–201 °C. For $C_{13}H_{14}N_2O_3S$ (278.3) calculated: 56.10% C, 5.07% H, 10.06% N; found: 55.88% C, 5.22% H, 9.97% N. IR: 1 500, 1 520, 1 585, 1 725. ^{1}H NMR: 2.36 s, 3 H (CH_3); 3.23 t, 2 H, *J* = 8 (H-4 or H-5); 3.79 t, 2 H, *J* = 8 (H-4 or H-5); 4.46 s, 2 H (CH_2N); 7.26 d, 2 H, *J* = 8 (arom. H); 8.00 d, 2 H, *J* = 8 (arom. H).

2-(4-Nitrobenzoylimino)thiazoline-3-acetic acid (**9h**). Yield 66%, m.p. 189–193 °C. For $C_{12}H_{11}N_3O_5S$ (309.3) calculated: 46.60% C, 3.58% H, 13.58% N; found: 46.67% C, 3.75% H, 13.72% N. IR: 1 530, 1 590, 1 615, 1 720. ^{1}H NMR: 3.29 t, 2 H, *J* = 8 (H-4 or H-5); 3.85 t, 2 H, *J* = 8 (H-4 or H-5); 4.51 s, 2 H (CH_2N); 8.30 m, 4 H (arom. H).

2-(4-Chlorobenzoylimino)-5-methoxy-2,3-dihydrothiazole-3-acetic acid (**12**). Yield 65%, m.p. 216–220 °C. For $C_{13}H_{11}ClN_2O_4S$ (326.8) calculated: 47.79% C, 3.39% H, 8.57% N; found: 48.01% C, 3.11% H, 8.63% N. IR: 1 180, 1 495, 1 605, 1 735. ^{1}H NMR: 3.86 s, 3 H (CH_3O); 4.96 s, 2 H (CH_2N); 7.07 s, 1 H (H-4); 7.53 d, 2 H, *J* = 8.5 (arom. H); 8.12 d, 2 H, *J* = 8.5 (arom. H).

Biology

Dextran and Ficoll–Paque were purchased from Pharmacia (Uppsala, Sweden); FMLP, PMA, cytochalasin B, ferricytochrome C, bovine serum albumin, superoxide dismutase, *Micrococcus lysodeicticus* and NADH were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); filters for the chemotactic chamber were from Millipore (Roma, Italy) and casein "Hammarsten" was from Merck (Darmstadt, Germany). Sterile 96-well microtitre plates with flat-bottomed wells were from Falcon Microtest III, Becton–Dickinson Labware, Milano, Italy. FMLP stock solution (10^{-2} mol/l in DMSO) was diluted before use in Krebs–Ringer-phosphate containing 0.1% glucose (KRPG), pH 7.4. PMA stock solution (1 mg/ml in DMSO) was diluted before use in KRPG. Casein stock solution containing 10 mg/ml in KRPG was diluted before use in KRPG containing 1 mg/ml bovine serum albumin (KRPG-A). Stock solutions (10^{-2} mol/l in DMSO) of the tested compounds and indomethacin were diluted before use in KRPG. Dimethyl sulfoxide did not interfere with any of the biological assays performed.

Preparation of Human Neutrophils

Cells were obtained from the blood of healthy subjects and neutrophils were purified employing the standard techniques of dextran sedimentation, centrifugation on Ficoll–Paque

and hypotonic lysis of contaminating red cells. The cells were washed twice, resuspended in KRPG at a final concentration of $50 \cdot 10^6$ cells/ml and kept at room temperature until used. The percentage of neutrophils was 98–100% pure and >99% viable as determined by Trypan blue exclusion test.

Random Locomotion

Random locomotion was evaluated using 48-well microchemotaxis chamber, by estimating the distance in micrometers which the leading-front of the cell migrated, using the method of Zigmond and Hirsch[19] after 90 min incubation at 37 °C. A 3 μm pore-size filter separated upper and lower compartments. The actual control for random locomotion was 40 ± 4 μm SE from 15 separate experiments.

Chemotaxis

Chemotaxis was studied by adding the chemoattractant to the lower compartment. The chemotactic factors used were casein (2 mg/ml in KRPG-A) and FMLP (10^{-8} mol/l in KRPG-A). Cells were incubated with 10^{-9}, 10^{-7} and 10^{-5} mol/l of the compounds for 10 min before the chemotactic assay. The actual control of chemotaxis induced by 10^{-6} M FMLP and 2 mg/ml casein was 78 ± 3 μm and 87.6 ± 2 μm, respectively (12 separate experiments done in triplicate).

Superoxide Anion Production

The O_2^- was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome[20] modified for microplate-based assays. The tests were carried out in a final volume of 200 μl containing $4 \cdot 10^5$ neutrophils, 100 nmol of cytochrome C and KRPG. At zero time, the stimulant was added and the plates were incubated into a microplate reader (Ceres 900, Bio-TeK instruments, INC) with the compartment T set at 37 °C. Absorbance was recorded at wavelengths of 550 and 468 nm. Differences in absorbance at the two wavelengths were used to calculate nmol of O_2^- produced, using a millimolar extinction coefficient for cytochrome C of 15.5. The stimulants employed were PMA (100 ng/ml in KRPG) and FMLP (10^{-6} mol/l in KRPG). Neutrophils were preincubated with cytochalasin B (5 μg/ml) 5 min prior the activation by FMLP. Assays were done in triplicate for each experimental condition. Cells were incubated with 10^{-9}, 10^{-7} and 10^{-5} mol/l of the compounds for 10 min before the addition of stimulus. The actual control of O_2^- generation by 10^{-6} M FMLP was 20 ± 2 nmol/$4 \cdot 10^5$ cells/5 min (12 separate experiments done in triplicate).

Enzyme Assay

Release of neutrophil granule enzymes was evaluated by determining lysozyme activity[20], modified for microplate-based assays. Cells were incubated with 10^{-9}, 10^{-7} and 10^{-5} mol/l of the compounds 10 min before the addition of stimulus. $3 \cdot 10^6$ cells were incubated in microplate wells in the presence of the stimulus for 15 min at 37 °C. The plates were then centrifuged for 5 min at 400 g and the lysozyme was quantified nephelometrically by the rate of lysis of cell wall suspension of *Micrococcus lysodeikticus*. Reaction rate was measured with a microplate reader at 465 nm. Enzyme was expressed as a net percentage of total enzyme content released by 0.1% Triton X-100. Total enzyme activity was 85 ± 1 μg/$1 \cdot 10^7$ cells/min.

The degranulating agents used were PMA 0.1 μg/ml and FMLP 10^{-6} mol/l, both in KRPG. The actual control of lysozyme release by 10^{-6} M FMLP and 100 ng/ml PMA was 45 ± 4% and 52 ± 5% of release/$3 \cdot 10^6$ cells/10 min, respectively (12 separate experiments done in triplicate). Cells were preincubated with cytochalasin B (5 μg/ml) 15 min prior the activation by FMLP. Assays were done in triplicate for each experimental condition.

Treatment of the Tested Compounds

When requested, neutrophils were preincubated for 10 min at room temperature with 10^{-9}, 10^{-7} and 10^{-5} M (benzoylimino)thiazoles or indomethacin before the incubation step for cell functionality.

Measurement of Enzymatic Activity

To assess possible cytotoxic effects of the tested compounds, the cytoplasmic marker enzyme, lactate dehydrogenase (LDH), was determined by measuring the rate of oxidation of NADH. The absorbance change was followed at 340 nm (ref.[21]).

Statistical Analysis

The nonparametric Wilcoxon test was used in the statistical evaluation of differences between groups.

This work was supported by a grant from the Italian National Research Council (CNR), MURST and Associazione Italiana per la Ricerca sul Cancro (AIRC). We are grateful to Banca del Sangue di Ferrara for supplying fresh blood and to Merck Sharp & Dohme (NJ, U.S.A.) for the gift of indomethacin. We thank Prof. E. Abignente (University of Naples) for the helpful discussion on 2-(benzoylimino)thiazole-3-acetate.

REFERENCES

1. Andreani A., Rambaldi M., Bonazzi D., Fabbri G., Greci L., Galatulas I., Bossa R.: *Arch. Pharm. (Weinheim, Ger.)* **1983**, *316*, 141.
2. Andreani A., Rambaldi M., Bonazzi D., Greci L.: *Boll. Chim. Farm.* **1980**, *119*, 647.
3. Tedeschi E. (Plantex Ltd., Israel): Ger. Offen. 2,505,068; *Chem. Abstr.* **1975**, *83*, 206269.
4. Abignente E., De Caprariis P., Fattorusso E., Mayol L.: *J. Heterocycl. Chem.* **1989**, *26*, 1875.
5. Drain D. J., Daly M. J., Davy B., Horlington M., Howes J. G. B., Scruton J. M., Selvay R. A.: *J. Pharm. Pharmacol.* **1970**, *22*, 684.
6. Abramson S., Korchak H., Ludewig R., Edelson E., Haines K., Levin R. I., Herman R., Rider L., Kimmel S., Weissmann G.: *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 7227.
7. Abramson S. B., Cherksey B., Gude D., Leszczynska-Piziak J., Philips M. R., Blau L., Weissmann G.: *Inflammation (N. Y.)* **1990**, *14*, 11.
8. Crowell R. E., Van Epps D. E.: *Inflammation (N. Y.)* **1990**, *14*, 163.
9. Coburn R. A., Glennon R. A.: *J. Heterocycl. Chem.* **1973**, *10*, 487.
10. Kaye I. A., Parris C. L.: *J. Org. Chem.* **1951**, *16*, 1761.
11. Schmelkes F. C., Rubin M.: *J. Am. Chem. Soc.* **1944**, *66*, 1631.

12. Douglass I. B., Dains F. B.: *J. Am. Chem. Soc.* **1934**, *56*, 719.
13. Viallet M.-P., Boucherle A., Cohen-Addad C.: *Eur. J. Med. Chem.* **1979**, *14*, 553.
14. Forlani L., Medici A., Todesco P. E.: *Tetrahedron Lett.* **1976**, 201.
15. Abramson S. B., Leszczynska-Piziak J., Clancy R. M., Philips M., Weissmann G.: *Biochem. Pharmacol.* **1994**, *47*, 563.
16. Kaplan H. B., Edelson H. S., Korchak H. M., Given W. P., Abramson S., Weissmann G.: *Biochem. Pharmacol.* **1984**, *33*, 371.
17. Abramson S. B., Weissmann G.: *Arthritis Rheum.* **1989**, *32*, 1.
18. Crouch S. P. M., Fletcher J.: *Infect. Immun.* **1992**, *60*, 4504.
19. Zigmond S. H., Hirsch J. G.: *J. Exp. Med.* **1973**, *137*, 387.
20. Torrini I., Pagani Zecchini G., Paglialunga Paradisi M., Lucente G., Mastropietro G., Gavuzzo E., Mazza F., Pochetti G., Traniello S., Spisani S.: *Biopolymers* **1996**, *39*, 327.
21. Bergmeyer H. U. in: *Methods of Enzymatic Analysis* (H. U. Bergmeyer, Ed.), 3rd ed., Vol. 3, p. 118. Verlag Chemie, Weinheim 1983.