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SCIENCE DIRECT
Bioorganic & Medicinal Chemistry 14 (2006) 5152–5160
Bioorganic & Medicinal Chemistry

Synthesis and biological evaluation of novel heterocyclic ionone-like derivatives as anti-inflammatory agents

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Received 31 January 2006; revised 29 March 2006; accepted 4 April 2006
Available online 8 May 2006

Abstract—Five- and six-membered heterocyclic ionone-like derivatives **4**–**6** have been synthesised in one step and with good yield from the key intermediate **3a** and appropriate bifunctional reagents. Four were active as inhibitors of the respiratory burst of human neutrophils without affecting cell viability. The two most active compounds (**5a**,**d**) tested in neutrophil migration assays, were also found to be potent inhibitors of neutrophil chemotactic responsiveness. These two molecules could be considered as lead compounds of new drugs which can be an effective tool to treat psoriasis and related neutrophilic dermatoses.

1. Introduction

Retinoic acid and its derivates have been used extensively in dermatological applications such as acne, psoriasis and keratinization disorders, and studied in many other anti-inflammatory applications.[1,2]

Unfortunately, their action has also been associated with a high incidence of undesirable side effects: skin and mucous membrane toxicity, hyperlipidemia, skeletal side effects, visual disturbance and teratogenicity.[3–7] Consequently, in an effort to both increase efficacy and reduce toxicity, a great number of retinoids have been synthesised. The original structure of vitamin A has been modified in the cyclohexenyl ring, the polyene side chain and the polar terminal group. Major transformations have led to compounds which barely resemble the original retinoic acid. The wide spectrum of effects which they produce suggests that they can interact with a diverse range of receptors. Among these, adapalene and tazarotene have been introduced on the market for topical use and 'short heteroretinoids' are currently studied as potent inducers of differentiation and apoptosis. Moreover, even if to a minor extent, retinoid-like compounds have been studied and patented as anti-inflammatory agents.[8–11] These so-called third generation of retinoids are often associated with reduced toxicity, while biological activity is maintained or even enhanced. Therefore, to design selective retinoids devoid of dangerous side effects with high biological activity is not too far from becoming reality.

Although almost all retinoid-like compounds have a carboxylic function, there are few examples in which this function is replaced by amides[12] or amines.[13,14] In many cases, the authors' statements regarding these compounds are very optimistic. For example, some say that amides maintain a noticeable activity both in vitro and in vivo and are much less toxic and teratogenic than retinoid acid itself. Nonetheless, these examples remain isolated in the vast panorama of retinoid and retinoic-like derivates.

We have already reported that heterocyclic ionone-like derivatives, which can be linked to the so-called 'short heteroretinoids', possess antimicrobial activity.[15] We would now like to demonstrate that this class of compounds is endowed with anti-inflammatory and histoprotective properties potentially useful in controlling neutrophilic inflammatory responses.[16–18]

Since major pathologies targeted by retinoic acid and retinoid-like compounds are represented by inflammatory dermatoses, such as acne and psoriasis, the

Keywords: Isoxazole; Pyrazole; Pyrimidine ionone-like derivatives; Antiinflammatory activity; Neutrophil superoxide; Neutrophil chemotaxis.
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doi:10.1016/j.bmc.2006.04.007

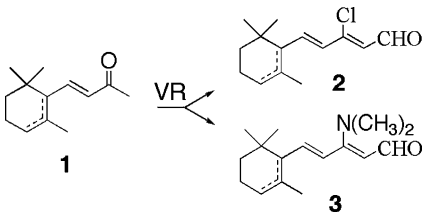
evaluation of the new compounds potential anti-inflammatory activity was tested using purified human neutrophils. Neutrophils play a crucial role in these diseases and are indeed recruited in inflamed skin by locally generated chemoattractants. In turn, extravasated neutrophils undergo activation with a production of oxidants and release of enzymes, favouring both the amplification of the inflammatory reaction and the development of tissue injury.[16,19] In the present study, the potential anti-inflammatory and histoprotective properties of our short heterocyclic ionone-like derivates were carried out by testing the ability of these cells to migrate and to undergo activation of the respiratory burst as measured by determining superoxide production (O_2^-).

2. Chemistry

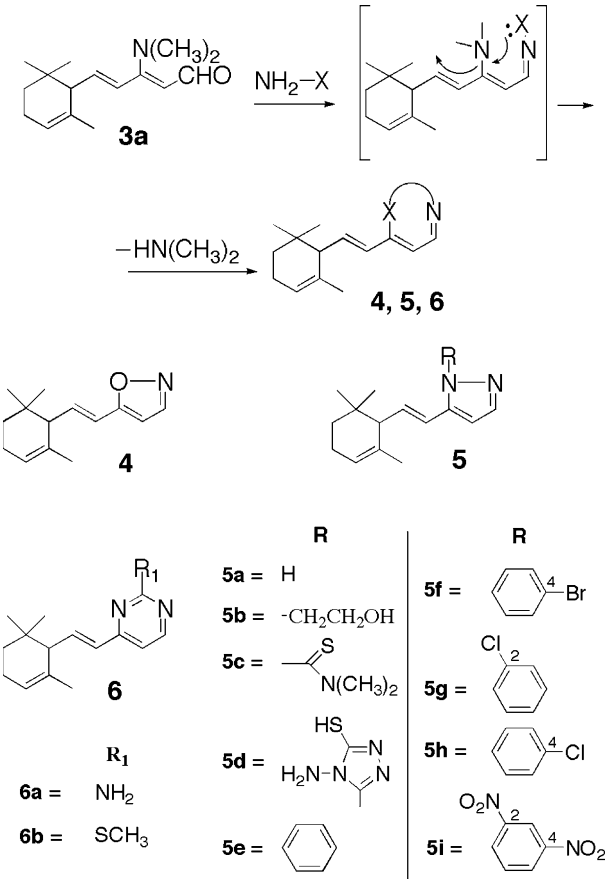
While studying the Vilsmeier reaction (VR) on α- and β-ionones **1**, we outlined both the formation of the classical β-chlorovinylaldehydes **2** and the unexpected products **3**, which is unprecedented in a Vilsmeier reaction (Scheme 1).

Somewhat surprisingly, the chlorine of compounds **2** had difficulty in reacting and consequently all attempts to cyclise those derivatives failed. We thus opted to examine a different approach, turning our attention to compound **3**.

We have already shown that enamines comparable to compound **3** exhibit a propensity to amine exchange, in particular when an electron-withdrawing group is present in β-position.[20] Moreover, cyclisation can occur and become the only product when a bifunctional reagent is used. Compound **3** have shown similar behaviour. In fact, the reaction of **3** and a number of bifunctional reagents allowed for the formation of a series of heterocycles ionone-like derivatives. We speculate that this reaction, which involves both the formyl and the dialkylamino groups, could be considered a general reaction in which the primary amine of the bifunctional reagent couples with the carbonyl group giving the corresponding imine, and secondly, via a concerted pathway, the other part of the reagent displaces the dialkylamino group which acts as an outstanding leaving group (Scheme 2). Nonetheless, particular care has to be taken in performing this reaction. In fact, since the reaction is very sensitive both to time and temperature, each reagent has its own story (see Section 5).



Scheme 1. Vilsmeier reaction on α- and β-ionones.



Scheme 2. Synthesis of heterocyclic ionone-like derivatives.

Since preliminary studies carried out in our laboratory showed that the derivatives coming from α-ionones were more easily obtainable and the activities were perfectly comparable, we focused our attention on them.

Refining and improving our method, we then resynthesised compounds **4**, **5a**,**e**,**i** and **6a**,**b**, bettering the yield, and prepared new derivatives **5b–d** and **5f–h**.

3. Biological results

3.1. Effect on neutrophil viability

In order to test the effects of the new compound on the cell survival, neutrophil viability was evaluated in the presence of different doses of the compounds by the ethidium bromide–fluorescein diacetate test. As a control, the cells were exposed to higher dose of vehicle EtOH alone.

The data (Table 1) represent means of two independent experiments. Only the compounds **5e** is cytotoxic.

3.2. Effect on neutrophil superoxide production

Ten compounds were tested for their effect on neutrophil's superoxide production triggered by 10 ng/mL TNF-α on fibrinogen-coated wells. Human neutrophils

Table 1. Effect of different doses (100 μM → 0.1 μM) of heterocyclic ionone-like derivatives and EtOH (vehicle) on cell viability

Compound	100 μM	10 μM	1 μM	0.1 μM
EtOH	98	—	—	—
4	—	100	99	98
5a	98	100	97	100
5b	—	99	95	94
5c	—	97	95	99
5d	—	100	100	100
5e	<70	—	—	—
5f	95	99	94	98
5g	95	99	94	98
5h	93	94	94	92
6a	93	94	92	94
6b	96	95	96	99

Results are expressed as percentage of viable cells.

(5×10^4) exposed to 10 ng/mL TNF-α were found to produce an average 3.5 ± 0.5 (mean ± 1 SD, $n = 3$) nmol of O_2^- as detected by superoxide dismutase-inhibitable reduction of ferrocytochrome *c*. Under the same experimental conditions, resting neutrophils generated 0.4 ± 0.2 (mean ± 1 SD, $n = 3$) nmol of O_2^-.

As shown in Figure 1, among the compounds examined only four compounds (**5a**,**d**,**g** and **6a**) were able to inhibit O_2^- production in a dose-dependent manner. Table 2 reports the IC_{50} for each of these compounds.

3.3. Effect on neutrophil chemotaxis

Compounds **5a** and **5d**, which became more effective on superoxide assay, were studied for their activity on neutrophil chemotaxis using fMLP as chemoattractant. *trans*-Retinoic acid, which possesses anti-inflammatory activity in several experimental models,[21] was also tested as reference. Neutrophils were incubated in the upper compartment of the chemotaxis chamber in the absence or in the presence of different doses of each compound. Their locomotory response to 10 nM fMLP in the lower compartment was tested after 45 min of incubation.

As shown in Figure 2 the chemotactic response of neutrophils was inhibited by compounds **5a** and **5d** in a dose-dependent manner. Data are expressed as net migration obtained subtracting spontaneous migration.

Table 2. IC_{50} (means ± 1SD, $n = 3$) of **5a**,**d**,**g** and **6a** on the production of O_2^- on human neutrophils

Compound	**5a**	**6a**	**5d**	**5g**
IC_{50} (μM)	3.01 ± 2.0	13.3 ± 7.4	2.85 ± 0.01	24.5 ± 0.4

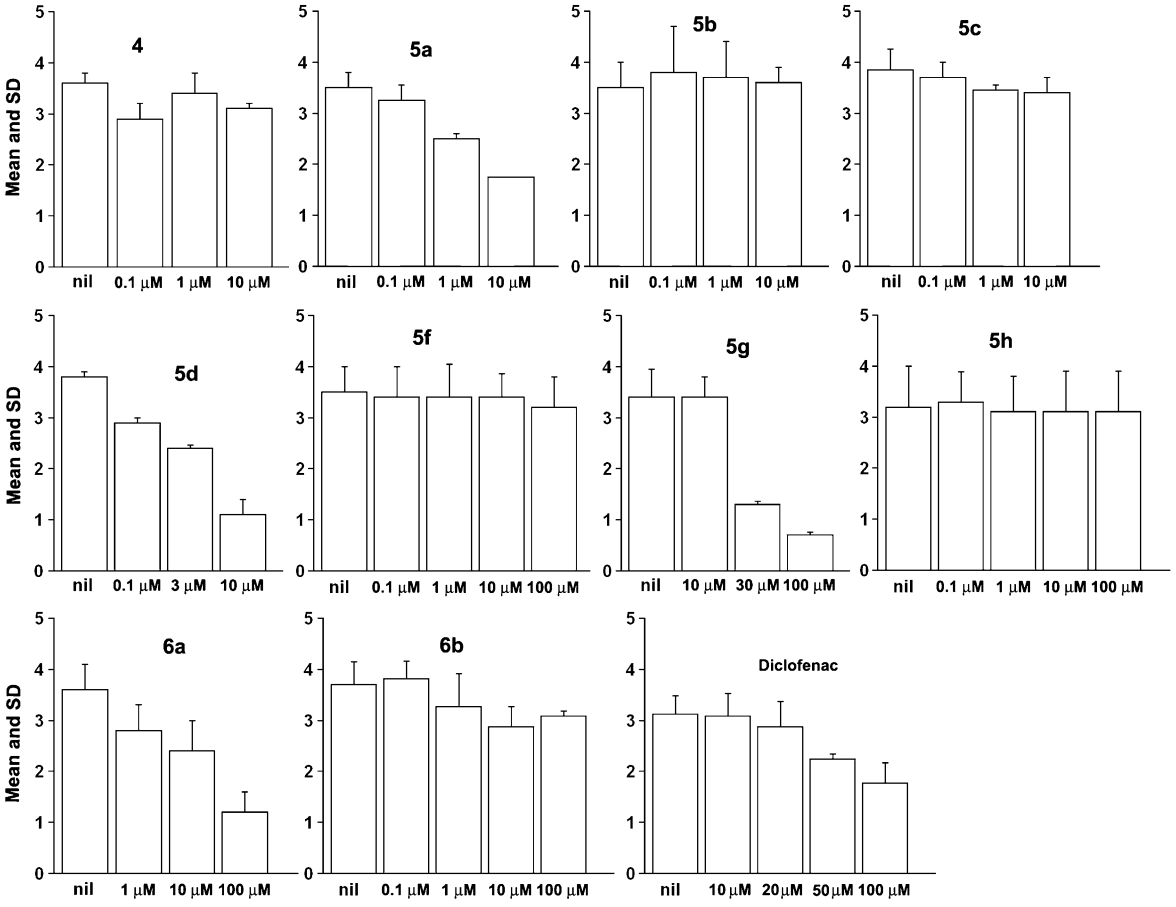


Figure 1. Effect of different doses (abscissa) of heterocyclic ionone-like derivatives on the superoxide anion production (O_2^-) in neutrophils adherent to a biologic substrate (fibrinogen) triggered with TNF-α (10 ng/mL). Resting values are not reported in figure.

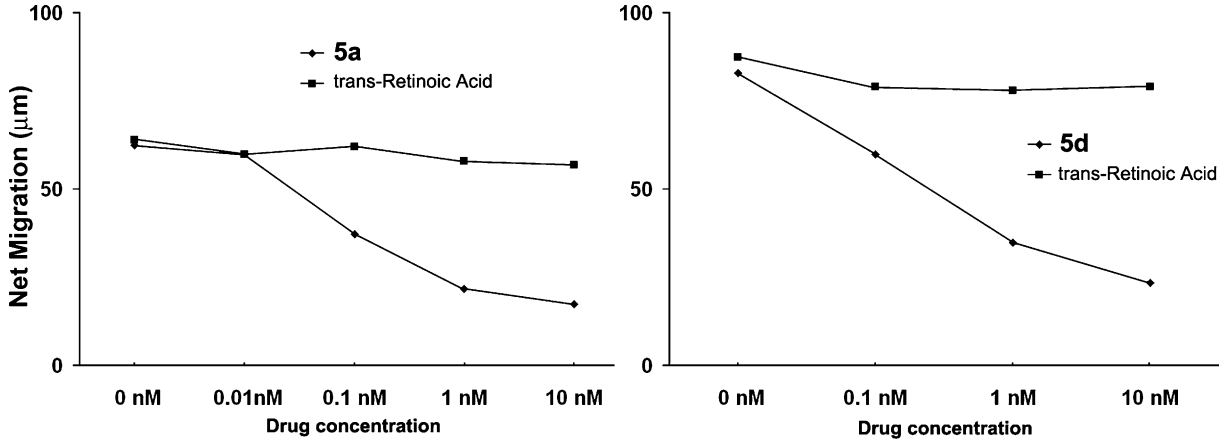


Figure 2. Dose-dependent inhibition of fMLP-induced neutrophil migration by different doses (abscissa) of **5a** and **5d** compared with *trans*-retinoic acid. Results are expressed as net migration (μm).

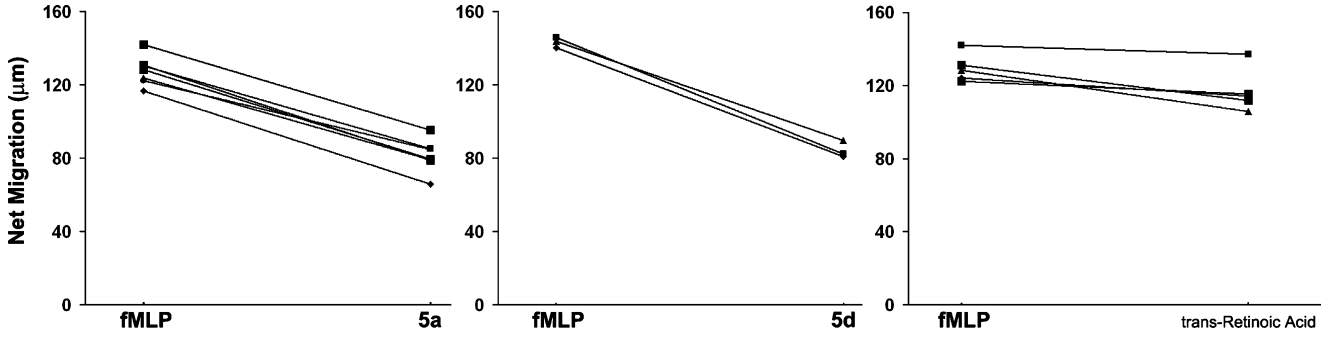


Figure 3. Effect on neutrophil chemotaxis to fMLP in the presence of derivatives **5a** (10 nM) and **5d** (10 nM) and *trans*-retinoic Acid 10 nM. Tests were carried out using neutrophils from different healthy volunteers. Statistical analysis: one-tailed P value is <0.01 for compound **5d** and < 0.001 for compound **5a**. (Mann–Whitney test) Differences were accepted as significant when $P < 0.05$.

The dose of compounds **5a** and **5d** able to induce 50% inhibition (IC_{50}) was 0.46 and 0.9 nM.

In contrast, retinoic acid had no inhibitory effect on neutrophil chemotaxis in same conditions.

Moreover, the effects of compounds **5a** and **5d** (10 nM) on chemotaxis were confirmed by using neutrophils from different donors. As shown in Figure 3, the chemotactic response of neutrophils versus fMLP was inhibited by equimolar concentrations (10 nM) of derivatives **5a** and **5d**, whereas *trans*-retinoic acid was ineffective.

4. Discussion and conclusion

The present data show that among eleven heterocyclic ionone-like derivatives tested, four are active as inhibitors of the respiratory burst of human neutrophils without affecting cell viability. Moreover, the two most active compounds, that is, **5a** and **5d**, were tested in neutrophil migration assays and were found to be potent inhibitors of neutrophil chemotactic responsiveness as well. In particular, inhibition of neutrophil chemotaxis could be detected at very low concentrations (i.e., 0.1 nM) of the compounds. This appears to be a relevant finding in as much as nonsteroidal anti-inflammatory drugs now available in clinical practice generally display inhibitory activities at micromolar concentrations, as far as neutrophil functional responses are concerned.[22,23] In addition, it also seems of interest that other related compounds such as *trans*-retinoid acid and the retinoic-like compound Adapalene are, respectively, inactive or active at concentrations three-order higher[21] than those herein found in our heterocyclic ionone-like derivatives. Therefore, these compounds appear to have features consistent with the possibility of interfering with the development of tissue injury during neutrophilic inflammatory reactions. For instance, it is known that an aromatic retinoid, Etretinate, is both active in skin diseases such as acne and psoriasis and in inhibiting in vivo neutrophil migration.[24,25] Similarly, and more importantly, our compounds might reduce not only the recruitment of neutrophils in skin during psoriasis or related neutrophilic dermatoses but might also directly reduce neutrophil ability to cause skin damage. Present compounds are indeed capable of inhibiting neutrophil respiratory burst, known to be instrumental in the development of oxidative tissue injury.[26] Moreover, it is noteworthy that this class of compounds also has direct anti-bacterial activity.[15] Taking into account that in certain inflammatory dermatoses, such as acne, the intervention of microorganisms is a crucial pathogenetic event, coupled with the consequent involvement of neutrophils, the present

findings are in agreement with the concept of retinoic-like compounds as a new strategy to simultaneously target cutaneus infection and inflammation with one single treatment.

From a structural–activity point of view it can be pointed out that the pyrazole ring and its substituents in position-1 play a fundamental role in determining and increasing the activity. In fact, the substitution of one nitrogen of the pyrazole with oxygen, which led to the isoxazole derivative **4**, resulted in a significant loss of activity. The introduction of a substituent in position-1 led to a general decrease of activity. Only the 2-chlorophenyl and the aminotriazole substituents were able to maintain and even increase the activity. The positive effect of the amino group seems confirmed by the good activity of **6a** which is the only nonpyrazole ring having significant activity. The high activity of **5d** (IC_{50} 2.85 μM), which sums in his structure the pyrazole and the amino group, which is in turn linked to the triazole ring, substantiates the promising characteristics of these groups which deserve further and more profound studies.

In conclusion, our synthetic method proved to be versatile enough to obtain ionone-like derivatives in which the heteroaromatic ring and their substituents can affect both the strength and the character of the biological activity, eventually addressing the activity towards different targets. In the present case, the two most active compounds **5a** and **5d** are good candidates for prototype drugs in psoriasis and related neutrophilic dermatoses.

5. Experimental

5.1. General

Melting points were determined with Fisher–Johns apparatus and are uncorrected. The IR spectra were recorded in film or in potassium bromide disks on a Perkin-Elmer 398 spectrometer. The ^1H and ^{13}C NMR spectra were recorded a Varian Gemini 200 (200 MHz, ^1H; 50 MHz, ^{13}C) spectrometers in deuteriochloroform solutions with tetramethylsilane as the internal standard ($\delta = 0$). The purity of all compounds was checked by thin-layer chromatography on silica gel 60-F-254 precoated plates and the spots were located in UV light or by vanillin in sulfuric acid. Elemental analyses were performed on a Carlo Erba 1106 Elemental Analyser in the Microanalysis Laboratory in our Department.

5.2. Synthesis of key intermediate (3a)

Phosphorus oxychloride (50.0 mmol, 4.57 mL) was added dropwise, for 15 min at 0 °C, to 3.87 mL of *N*,*N*-dimethylformamide in a two-necked flask protected from atmospheric moisture and efficiently stirred with a magnetic bar. A solution of α-ionone (25.0 mmol) in 3 mL of dimethylformamide was added dropwise into the above Vilsmeier reagent cooled at −20 °C. The reaction mixture was allowed to stir while the temperature rose to 0 °C in 45 min, then poured onto crushed ice. The aqueous layer was separated, alkalinized (neutralized for **2**) with a diluted solution of sodium hydroxide and allowed to stand overnight at room temperature. The resulting water–oil mixture led to an oil which solidified on standing, furnishing **3a** [3-dimethylamino-5-(2,6,6-trimethyl-2-cyclohexen-1-yl)-2,4-pentadienal] as an essentially pure pale yellow powder (40% yield); mp 147 °C from *n*-hexane.[27]

5.3. Synthesis of 4, 5a–i and 6a–b

5.3.1. 5-(2,6,6-Trimethyl-2-cyclohexen-1-yl)ethenyl-isoxazole (4). To a solution of **3a** (0.4 g, 1.6 mmol) in 10 mL of ethanol, 0.5 g (7.0 mmol) of hydroxylamine hydrochloride dissolved in 3 mL of water and 2 mL of a 10% water solution of sodium hydroxide was added portionwise. The mixture was allowed to stand at room temperature for 3 days. The resulting mixture was added with 10 mL of water and extracted with chloroform. The combined chloroform extracts were washed with brine, dried and evaporated under reduced pressure. The residue was purified by chromatography on silica gel (toluene/ethyl acetate 1:1), giving **4** in 90% yield as a thick oil.

IR (film): *ν* 3100, 2900, 1640, 1570, 1449, 1370, 915, 870 cm^{-1}; ^1H NMR (200 MHz): δ 0.82 (3H, s, CH_3), 0.91 (3H, s, CH_3), 1.22 (1H, m, H-5), 1.42 (1H, m, H-5), 1.56 (3H, s, CH_3), 2.01 (2H, m, H-4), 2.23 (1H, d, H-1 $J = 9.50$ Hz), 5.46 (1H, m, H-3), 5.85 (1H, dd, ethene, $J = 9.50$; 15.23 Hz), 6.28 (1H, d, ethene, $J = 15.23$ Hz), 6.32 (1H, d, CH), 7.45 (1H, d, CH); ^{13}C NMR (50 MHz): δ 23.3 (CH_3), 24.5 (CH_2), 27.2 (CH_3), 28.4 (CH_3), 31.6 (CH_2), 32.9 (C), 55.2 (CH), 93.1 (CH), 121.1 (CH), 122.3 (CH), 133.2 (CH), 133.2 (C), 142.4 (CH), 158.01 (C). Anal. Calcd for $C_{14}H_{19}NO$: C, 77.38; H, 8.81; N, 6.45; O, 7.36. Found: C, 76.99; H, 8.90; N, 6.22.

5.3.2. 5-(2,6,6-Trimethyl-2-cyclohexen-1-yl)ethenyl-1*H*-pyrazole (5a). Hydrazine hydrate (1 mL, 20 mmol) was added in a single portion to a solution of **3a** (0.25 g, 1 mmol) in 10 mL of ethanol and the mixture was stirred at room temperature for 30 min and was allowed to stand at −10 °C for 2 days. After evaporation to dryness, the residue was purified by chromatography on silica gel (toluene/ethyl acetate 1:1) giving **5a** in 89% yield as thick oil.

IR (film): *ν* 3200, 2915, 1650, 1560, 1500, 1380, 1360 cm^{-1}; ^1H NMR (200 MHz): δ 0.86 (3H, s, CH_3), 0.91 (3H, s, CH_3), 1.22 (1H, m, H-5), 1.45 (1H, m, H-5), 1.62 (3H, s, CH_3), 2.05 (2H, m, H-4), 2.28 (1H, d, H-1, $J = 9.80$ Hz), 5.48 (1H, m, H-3), 6.05 (1H, dd, ethene, $J = 15.68$ Hz and $J = 9.80$ Hz), 6.32 (1H, d, CH), 6.38 (1H, d, ethene, $J = 15.68$ Hz), 7.51 (1H, d, CH), 14.4 (1H, s, NH); ^{13}C NMR (50 MHz): δ 23.5, 23.6, 27.4, 28.3, 32.0, 33.0, 55.3, 102.6, 121.2, 121.9, 134.1, 134.4, 135.1, 146.7. Anal. Calcd for $C_{14}H_{20}N_2$: C, 77.73; H, 9.32; N, 12.95. Found: C, 77.44; H, 9.11; N, 13.02.

5.3.3. 2-{5-[2-(2,6,6-Trimethyl-2-cyclohexen-1-yl)ethenyl]-1*H*-pyrazol-1-yl}-ethanol (5b). A solution of 2-

hydrazinoethanol (0.14 mL, 2 mmol) in 1 mL of ethanol was added in a single portion to a solution of **3a** (0.5 g, 2 mmol) in 10 mL of ethanol. The mixture was allowed to stir at room temperature until the starting product had almost disappeared (control by TLC, 15 days). After evaporation to dryness, the residue was chromatographed on silica gel eluting first with toluene and secondly with toluene/ethyl acetate 1:1. The second eluate gave **5b** in 60% yield as a thick oil.

IR (film) ν 3350, 2900, 1630, 1450, 1380, 1360 cm^{-1}; ^1H NMR: δ 0.87 (3H, s, CH_3), 0.93 (3H, s, CH_3), 1.22 (1H, m, H-5), 1.48 (1H, m, H-5), 1.61 (3H, s, CH_3), 2.03 (2H, m, H-4), 2.25 (1H, d, H-1, J = 8.98 Hz), 2.60 (1H, s, OH, deuterium oxide exchangeable), 3.98 (2H, m, CH_2), 4.18 (2H, m, CH_2), 5.48 (1H, m, H-3), 6.04 (1H, dd, ethene, J = 8.98 Hz, J = 15.05 Hz), 6.30 (1H, d, CH), 6.35 (1H, d, ethene, J = 15.05 Hz), 7.33 (1H, d, CH); ^{13}C NMR (50 MHz) δ 23.40 (CH_3), 23.47 (CH_2), 27.40 (CH_3), 28.20 (CH_3), 31.95 (CH_2), 32.94 (C), 54.06 (CH_2), 55.13 (CH), 62.29 (CH_2), 102.54 (CH), 121.68 (CH), 122.36 (CH), 133.78 (CH), 134.40 (C), 139.30 (CH), 151.92 (C). Anal. Calcd for $C_{16}H_{24}N_2O$: C, 73.81; H, 9.29; N, 10.76, O, 6,14. Found: C, 74.11; H, 9.51; N, 10.43.

5.3.4. *N*,*N*-Dimethyl-5-[(2,6,6-trimethyl-2-cyclohex-1-yl)ethenyl]-1*H*-pyrazole-1-carbothioamide (5c). A mixture of **3a** (0.5 g, 2 mmol) and thiosemicarbazide (0.21 g, 2,3 mmol) in 10 mL of ethanol was allowed to stir for 20 days at room temperature (reaction monitored on TLC). After evaporation to dryness, the residue was chromatographed on silica gel eluting first with toluene and second with toluene/ethyl acetate 1:1. The crude product obtained from the second eluate was purified by silica gel chromatography (toluene/ethyl acetate 1:1): red oil, yield 51%.

IR (film) ν 2900, 2830, 1520, 1450, 1390, 1370, 1280, 1100 cm^{-1}; ^1H NMR: δ 0.86 (3H, s, CH_3), 0.92 (3H, s, CH_3), 1.22 (1H, m, H-5), 1.42 (1H, m, H-5), 1.61 (3H, s, CH_3), 2.05 (2H, m, H-4), 2.20 (1H, d, CH), 3.01 (3H, s, CH_3), 3.54 (3H, s, CH_3), 5.48 (1H, m, H-3), 6.12 (1H, dd, ethene), 6.37 (1H, d, CH), 6.50 (1H, d, ethene), 7.52 (1H, d, CH); ^{13}C NMR (50 MHz) δ 23.46 (CH_3), 23.54 (CH_2), 27.38 (CH_3), 28.21 (CH_3), 32.00 (CH_2), 33.11 (C), 42.96 (CH_3), 44.38 (CH_3), 55.30 (CH), 104.55 (CH), 118.86 (CH), 122.18 (CH), 133.75 (C), 136.99 (CH), 140.84 (CH), 142.87 (C), 180.37 (C). Anal. Calcd for $C_{17}H_{25}N_3S$: C, 67.28; H, 8.30; N, 13.85; S, 10.57. Found: C, 67.58; H, 8.22; N, 13.57; S, 10.28.

5.3.5. 4-Amino-5-{5-[2-(2,6,6-trimethyl-2-cyclohexen-1-yl)ethenyl]pyrazol-1-yl}-4*H*-[1,2,4]triazole-3-thiol (5d). To a stirring solution of **3a** (0.5 g, 2 mmol) in 10 mL of ethylene glycol, 0.29 g (2 mmol) of Purpald® and 2 N NaOH (3 mL) were added portionwise. The solution changed colour from green to red. After 24 h of stirring at room temperature, the reaction was treated dropwise with a dilute solution of HCl until the solution tested was neutral. The resulting yellow solid was filtered, washed with water, dried and crystallized from cyclohexane: yield 65%; mp 138–139 °C.

IR (KBr) ν 3300, 2930, 2830, 1650, 1600, 1490, 1450, 1360 cm^{-1}; ^1H NMR: δ 0.85 (3H, s, CH_3), 0.92 (3H, s, CH_3), 1.22 (1H, m, H-5), 1.48 (1H, m, H-5), 1.59 (3H, s, CH_3), 2.04 (2H, m, H-4), 2.30 (1H, d, H-1), 5.38 (2H, s, NH_2, deuterium oxide exchangeable), 5.48 (1H, m, H-3), 6.20 (1H, dd, ethene, J = 8.60 Hz, J = 15.05 Hz), 6.45 (1H, d, ethene, J = 15.05 Hz), 6.54 (1H, d, CH), 7.75 (1H, d, CH), 11.5 (1H, br s, SH, deuterium oxide exchangeable); ^{13}C NMR (50 MHz) δ 23.38 (CH_3), 23.51 (CH_2), 27.32 (CH_3), 28.25 (CH_3), 31.89 (C), 33.06 (CH_2), 55.32 (CH), 105.00 (CH), 112.35 (C), 117.10 (CH), 122.60 (CH), 133.23 (C), 135.70 (C), 139.64 (CH), 143.88 (CH), 145.14 (C). Anal. Calcd for $C_{16}H_{22}N_6S$: C, 58.15; H, 6.71; N, 25.43; S, 9.70. Found: C, 58.16; H, 6.84; N, 25.15; S, 9.45.

5.3.6. 1-Phenyl-5-(2,6,6-trimethyl-2-cyclohex-1-yl)ethenyl-1*H*-pyrazole (5e). Phenylhydrazine hydrochloride [0.5 g (3.2 mmol) in 5 mL of water and 0.8 g of sodium acetate trihydrate] was added to a stirred solution of **3a** (0.4 g,1.6 mmol) in 10 mL of ethanol. After 15 min stirring at reflux, the reaction mixture was allowed to stand at room temperature for 1 day. The mixture was poured into ice-water and the resulting oil was extracted with chloroform. The combined extracts were washed with brine, dried and concentrated. The red oil was purified by chromatography on silica gel (toluene/ethyl acetate 1:1): thick oil; yield 85%.

IR (film): ν 3100, 2920, 1670, 1600, 1500, 1450, 1390 cm^{-1}; ^1H NMR (200 MHz): δ 0.88 (3H, s, CH_3), 0.92 (3H, s, CH_3), 1.20 (1H, m, H-5), 1.45 (1H, m, H-5), 1.60 (3H, s, CH_3), 2.03 (2H, m, H-4), 2.25 (1H, d, H-1, J = 8.98 Hz), 5.45 (1H, m, H-3), 6.07 (1H, dd, ethene, J = 8.98; 15.76 Hz), 6.20 (1H, d, ethene, J = 15.76 Hz), 6.47 (1H, d, CH), 7.47 (5H, m, ArH) 7.61 (1H, d, CH); ^{13}C NMR (50 MHz) δ 23.44 (CH_3), 23.53 (CH_2), 27.39 (CH_3), 28.23 (CH_3), 32.00 (CH_2), 33.11 (C), 55.35 (CH), 104.37 (CH), 119.44 (CH), 122.17 (CH), 125.72 (CH), 125.72 (CH), 128.17 (CH), 129.50 (CH), 129.50 (CH), 133.69 (C), 136.62 (CH), 140.17 (C), 140.56 (CH), 141.71 (C). Anal. $C_{20}H_{24}N_2$: C, 82.15; H, 8.27; N, 9.58. Found: C, 81.86; H, 8.01; N, 9.82.

5.3.7. 1-(4-Bromophenyl)-5-(2,6,6-trimethyl-2-cyclohex-1-yl)ethenyl-1*H*-pyrazole (5f). 4-Bromophenylhydrazine hydrochloride (0.45 g, 2 mmol) in 3 mL of water followed by 1 mL of NaOH 10% w/v was added to a stirring solution of **3a** (0.5 g, 2 mmol) in 10 mL ethanol. The reaction was monitored with TLC. After 24 h of stirring at room temperature, ice-water was added to the reaction and extracted with chloroform. The combined extracts were washed with brine, dried and evaporated under reduced pressure. The residue was purified by silica gel chromatography eluting first with toluene and second with toluene/ethyl acetate 9:1. The second eluate gave **5f** as a red oil, yield 68%.

IR (film): *v* 3100, 2920, 2860, 1890, 1670, 1600, 1590, 1490, 1380, 1360 cm^{-1}; ^1H NMR (200 MHz): δ 0.87 (3H, s, CH_3), 0.92 (3H, s, CH_3), 1.25 (1H, m, H-5), 1.44 (1H, m, H-5), 1.59 (3H, s, CH_3), 2.05 (2H, m, H-4), 2.20 (1H, d, H-1, $J = 8.60$ Hz), 5.48 (1H, m, H-3), 6.12 (2H, m, ethene), 6.45 (1H, d, CH), 7.38 (2H, d, ArH) 7.58 (2H, d, ArH), 7.59 (1H, d, CH); ^{13}C NMR (50 MHz) δ 23.44 (CH_3), 23.52 (CH_2), 27.36 (CH_3), 28.26 (CH_3), 31.94 (CH_2), 33.14 (C), 55.35 (CH), 104.80 (CH), 119.06 (CH), 121.83 (C), 122.35 (CH), 127.08 (2CH), 132.64 (2CH), 133.50 (C), 137.24 (CH), 139.84 (C), 140.94 (CH), 141.78 (C). Anal. $C_{20}H_{23}N_2Br$: C, 64.69, H, 6.24; Br, 21.52; N, 7.54. Found: C, 64.82, H, 6.35; Br, 21.23; N, 7.68.

5.3.8. 1-(2-Chlorophenyl)-5-(2,6,6-trimethyl-2-cyclohex-1-yl)ethenyl-1*H*-pyrazole (5g). 2-Chlorophenylhydrazine hydrochloride (0.36 g, 2 mmol) in 3 mL of water followed by 1 mL of NaOH 10% w/v was added to a stirring solution of **3a** (0.5 g, 2 mmol) in 10 mL ethanol. After 28 h of stirring at room temperature, the reaction, which was monitored by TLC, was poured into ice-water and extracted with chloroform. The combined extracts were washed with brine, dried and evaporated under reduced pressure. The oil residue was purified by silica gel chromatography eluting first with toluene and second with toluene/ethyl acetate 9:1. The second eluate gave **5g** as a red oil, yield 70%.

IR (film): *v* 2900, 2860, 1890, 1670, 1590, 1490, 1380, 1360 cm^{-1}; ^1H NMR (200 MHz): δ 0.78 (3H, s, CH_3), 0.86 (3H, s, CH_3), 1.25 (1H, m, H-5), 1.43 (1H, m, H-5), 1.53 (3H, s, CH_3), 2.01 (2H, m, H-4), 2.10 (1H, d, H-1, $J = 8.60$ Hz), 5.48 (1H, m, H-3), 5.90 (1H, m, ethene), 6.47 (1H, d, CH), 7.43 (3H, m, ArH), 7.56 (1H, m, ArH), 7.65 (1H, d, CH,); ^{13}C NMR (50 MHz) δ 23.25 (CH_3), 23.47 (CH_2), 27.35 (CH_3), 27.95 (CH_3), 32.07 (CH_2), 32.90 (C), 55.37 (CH), 103.18 (CH), 118.48 (CH), 122.01 (CH), 127.95 (CH), 130.45 (CH), 130.78 (CH), 131.11 (C), 131.28 (C), 133.71 (CH), 136.72 (CH), 137.10 (C), 140.95 (CH), 141.89 (C). Anal. $C_{20}H_{23}N_2Cl$: C, 73.49; H, 7.09; Cl, 10.85; N, 8.57. Found: C, 73.42; H, 7.15; Cl, 10.66; N, 8.70.

5.3.9. 1-(4-Chlorophenyl)-5-(2,6,6-trimethyl-2-cyclohex-1-yl)ethenyl-1*H*-pyrazole (5h). 4-Chlorophenylhydrazine hydrochloride (0.36 g, 2 mmol) in 3 mL of water followed by 1 mL of NaOH 10% w/v was added to a stirring solution of **3a** (0.5 g, 2 mmol) in 10 mL ethanol. Following the above procedure the second eluate gave **5h** as a red oil, yield 69%.

IR (film): *v* 2900, 2860, 1890, 1670, 1590, 1490, 1380, 1360 cm^{-1}; ^1H NMR (200 MHz): δ 0.88 (3H, s, CH_3), 0.92 (3H, s, CH_3), 1.25 (1H, m, H-5), 1.43 (1H, m, H-5), 1.60 (3H, s, CH_3), 2.01 (2H, m, H-4), 2.20 (1H, d, H-1, $J = 8.60$ Hz), 5.48 (1H, m, H-3), 6.12 (2H, m, ethene), 6.46 (1H, d, CH), 7.43 (4H, m, ArH), 7.59 (1H, d, CH); ^{13}C NMR (50 MHz) δ 23.44 (CH_3), 23.52 (CH_2), 27.36 (CH_3), 28.26 (CH_3), 31.94 (CH_2), 33.13 (C), 55.35 (CH), 104.74 (CH), 119.07 (CH), 122.34 (CH), 126.80 (2CH), 129.67 (2CH), 133.50 (C), 133.91 (C), 137.19 (CH), 138.20 (C), 140.88 (CH), 141.79 (C). Anal. $C_{20}H_{23}N_2Cl$: C, 73.49; H, 7.09; Cl, 10.85; N, 8.57. Found: C, 73.40; H, 7.19; Cl, 10.74; N, 8.65.

5.3.10. 1-(2,4-Nitrophenyl)-5-(2,6,6-trimethyl-2-cyclohex-1-yl)ethenyl-1*H*-pyrazole (5i). A solution of 0.25 g (1 mmol) of **3a** and 2,4-dinitrophenylhydrazine [0.2 g (1 mmol) in ethanol/H_2SO_4] in 10 mL of ethanol was allowed to stand at −10 °C and 2 h at room temperature. The precipitated solid was collected by filtration and washed with ethanol, yielding **5i** as already pure yellow crystals (90% yield, mp 116–117 °C from ethanol).

IR (KBr): *v* 3100, 2900, 2860, 1610, 1530, 1450, 1390, 1350 cm^{-1}; ^1H NMR (200 MHz): δ 0.86 (3H, s, CH_3), 0.91 (3H, s, CH_3), 1.22 (1H, m, H-5′), 1.42 (1H, m, H-5), 1.58 (3H, s, CH_3), 2.05 (2H, m, H-4), 2.20 (1H, d, H-1, $J = 8.6$ Hz), 5.48 (1H, m, H-3), 6.12 (2H, m, ethene), 6.54 (1H, d, CH), 7.68 (1H, d, CH), [7.75 (1H, d), 8.56 (1H, dd), 8.85 (1H, d) ArH]; ^{13}C NMR (50 MHz): δ 23.42 (CH_3), 23.50 (CH_2), 27.30 (CH_3), 28.30 (CH_3), 31.85 (CH_2), 33.20 (C), 55.41 (CH), 105.94 (CH), 116.92 (CH), 121.61 (CH), 122.87 (CH), 127.74 (CH), 130.30 (CH), 133.00 (C), 138.11 (C), 139.84 (CH), 143.10 (C), 143.44 (CH), 146.02 (C), 147.00 (C). Anal. Calcd for $C_{20}H_{22}N_4O_4$: C, 62.82; H, 5.80; N, 14.65; O, 16.74. Found: C, 62.80; H, 5.71; N, 14.59.

5.3.11. 6-(2,6,6-Trimethyl-2-cyclohexen-1-yl)ethenyl-2-aminopyrimidine (6a). A solution of 0.5 g (2.0 mmol) of **3a** and guanidine carbonate [0.36 g (2.0 mmol) in 3 mL of water] in 30 mL of ethanol was refluxed for 48 h. The resulting oil was extracted with chloroform. The combined extracts were dried (sodium sulfate) and evaporated to afford an oil which was purified by chromatography on silica gel (toluene/ethyl acetate 1:1), giving **6a** in 85% yield as a thick oil.

IR (film): *v* 3300, 3180, 2900, 2860,1680, 1560, 1450 cm^{-1}; ^1H NMR (200 MHz): δ 0.87 (3H, s, CH_3), 0.93 (3H, s, CH_3), 1.25 (1H, m, H-5), 1.45 (1H, m, H-5), 1.59 (3H, s, CH_3), 2.04 (2H, m, H-4′), 2.30 (1H, d, H-1′ $J = 8.98$ Hz), 5.17 (2H, s, NH_2), 5.48 (1H, m, H-3), 6.20 (1H, d, ethene, $J = 8.60$ Hz), 6.58 (1H, d, ArH), 6.65 (1H, dd, ethene, $J = 15.05$, 8.60 Hz), 8.19 (1H, d, ArH); ^{13}C NMR (50 MHz,): δ 23.4 (CH_3), 23.6 (CH_2), 27.4 (CH_3), 28.4 (CH_3), 31.8 (CH_2), 33.1 (C), 55.1 (CH), 108.9 (CH), 122.5 (CH), 130.2 (CH), 133.3 (C), 141.6 (CH), 158.6 (CH),163.5 (C), 164.4 (C). Anal. Calcd for $C_{15}H_{21}N_3$: C, 74.03; H, 8.70; N, 17.27. Found: C, 73.82; H, 8.61; N, 17.11.

5.3.12. 2-Methylthio-4-[(2,6,6-trimethyl-2-cyclohexen-1-yl)ethenyl]-pyrimidine (6b). To the solution of 0.5 g (2.0 mmol) of **3a** in 10 mL of ethanol, 0.55 g (2.0 mmol) of *S*-methylisothiourea hydrogen sulfate dissolved in 2 mL of sodium hydroxide 2 N and 10 mL of water was added and the mixture refluxed for 48 h. The resulting oil was extracted with chloroform. The combined extracts were dried (sodium sulfate) and evaporated to afford an oil which was purified by chromatography on silica gel (toluene/ethyl acetate 1:1), giving **6b** in 85 % yield as a thick yellow oil.

IR (film): ν 3100, 2900, 2860,1680, 1560, 1450, 1375, 1200 cm^{-1}; ^1H NMR (200 MHz): δ 0.85 (3H, s, CH_3), 0.92 (3H, s, CH_3), 1.24 (1H, m, H-5), 1.44 (1H, m, H-5), 1.59 (3H, s, CH_3), 2.04 (2H, m, H-4), 2.27 (1H, d, H-1, $J = 8.98$ Hz), 2.29 (3H, s, SCH_3), 5.47 (1H, m, H-3), 6.19 (1H, d, ethene, $J = 8.65$ Hz), 6.59 (1H, d, ArH), 6.70 (1H, dd, ethene, $J = 15.15$, 8.65 Hz), 8.15 (1H, d, ArH); ^{13}C NMR (50 MHz): δ 18.95 (CH_3S), 23.41 (CH_3), 23.56 (CH_2), 27.81 (CH_3), 28.35 (CH_3), 31.78 (CH_2), 33.11 (C), 55.18 (CH), 108.82 (CH), 122.53 (CH), 130.17 (CH), 133.27 (C), 141.89 (CH), 158.34 (CH),164.408 (C), 164.55 (C). Anal. Calcd for $C_{16}H_{22}N_2S$: C, 70.03; H, 8.08; N, 10.21; S, 11.68. Found: C, 69.09; H, 7.78; N, 10.00; S, 11.29

5.4. Biological assay

5.4.1. Neutrophils. Heparinized (heparin 10 U/mL) venous blood was obtained from healthy volunteers. Neutrophils were isolated by dextran sedimentation, and subsequent centrifugation on a Ficoll–Hypaque density gradient, as described.[28]

Contaminating erythrocytes were removed by hypotonic lysis.[28] Neutrophils resuspended in incubation medium were >97% pure, as determined by morphologic analysis of Giemsa-stained cytopreps.

5.4.2. Neutrophil membrane integrity assay. Neutrophil viability measured as integrity of membrane was assessed according to Dankberg and Persidsky.[29] Briefly, cells (4×10^4/100 μL) were mixed with 50 μL staining solution (2 μg/mL fluorescein diacetate, 4 μg/mL ethidium bromide in HBSS) and incubated for 10 min at room temperature. Thereafter, a drop of cell suspension was placed on a slide, sealed with a coverslip and analysed under ultraviolet light in a dark field illumination. Neutrophils with intact membrane (i.e., viable cells) appeared as green fluorescent cells, whereas neutrophils with damaged and ethidium bromide-permeable membrane (i.e., necrotic cells) displayed a fluorescent red nucleus.

5.4.3. Superoxide anion assay. The production of superoxide anion O_2^- by neutrophils was measured as SOD-inhibitable reduction of cytochrome *c*, using a microplate reader (Titertek Twinreader Plus, Flow Lab, Ltd, Irvine, Scotland).[26] The assay was carried out in 96-well, flat-bottomed, polystyrene plates (Primaria plates, Falcon, Becton Dickinson, Oxnard, CA, USA). The wells were pre-treated with Fibrinogen by incubation in 5% CO_2 at 37 °C for 2 h. Immediately after washing, incubation medium containing 15 nmol cytochrome *c* was added to each well and the temperature brought to 37 °C in the microplate reader (usually within 15 min). The appropriate doses of each compound and the stimulus (TNF-α 10 ng/mL) were added followed by cells (5×10^4 neutrophils). This number of neutrophils per well was on the linear portion of the dose–response curve for this system. Experiments were carried out in triplicate, in presence and absence of SOD. The reduction of cytochrome *c* was monitored (3 h) at intervals (15 min) by reading the plate at 550 nm.

The amounts of O_2^- produced by neutrophils were determined from OD_{550} of sample without SOD minus the OD_{550} of matched samples with SOD, using an extinction coefficient of $0.0095\times 10^6\ M^{-1}$ calculated according to Leslie.[30]

5.4.4. Neutrophil incubation and chemotaxis assays. Neutrophils were incubated in absence and presence of appropriate doses of each compound or medium alone for 15 min in air at 37 °C temperature and then, washed and resuspended in medium to test their chemotactic activity. Neutrophil chemotaxis was assessed in a 48-well micro chemotaxis chamber (from Neuro Probe Inc., Gaithersburg, MD) using: a 3 μm pore size, 150 μm-thick cellulose ester filter.[31]

In the assay with cellulose filter, 10 nM *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) or control solution medium was seeded into the lower wells, while the upper wells were filled with 50 μL of untreated or short time incubated neutrophil suspension (2×10^6/mL). The whole chamber was incubated at 37 °C in air with 5% CO_2 for 45 min and, at the end, the filters were removed, fixed in ethanol, stained with Harris haematoxylin, dehydrated, cleared with xylene and mounted in Eukitt (Kindler, Freiburg, Germany). All treatments were performed in duplicate wells and the distance (micrometers) travelled by the leading front of cells was measured at 500× magnification, reading five randomly chosen fields for each filter. Data are expressed in net migration that was determined by subtracting neutrophils spontaneous migration to medium from the distance travelled by neutrophils toward the ligand.

Acknowledgment

Financial support of this research by the MIUR (program 2002) is gratefully acknowledged.

References and notes

1. Camisa, C.; Eisenstat, B.; Ragaz, A.; Weissmann, G. *J. Am. Acad. Dermatol.* **1982**, *6*, 620.
2. Orfanos, C. E.; Zouboulis, C. C.; Almond-Roesler, B.; Geilen, C. C. *Drugs* **1997**, *53*, 358.
3. Boehm, M. F.; Zhang, L.; Zhi, L.; McClurg, M. R.; Berger, E.; Wagoner, M.; Mais, D. E.; Suto, C. M.; Davies, J. A.; Heyman, R. A.; Nadzan, A. M. *J. Med. Chem.* **1995**, *38*, 3146.
4. Kamm, J. J.; Ashenfelter, K. O.; Ehmann, C. W. In Sporn, M. B., Roberts, A. B., Goodman, D. S., Eds.; The Retinoids; Academic Press: Orlando, FL, 1984; pp 288–326.
5. Amstrong, R. B.; Ashenfelter, K. O.; Eckhoff, C.; Levin, A. A.; Shapio, S. S. In *The Retinoid* 2nd ed.; Sporn, M. B., Roberts, A. B., Goodman, D. S., Eds.; Raven Press: New York, 1994; pp 545–572.
6. Standeven, A. M.; Beart, R. L.; Johnson, A. T.; Boehm, M. F.; Escobar, M.; Heyman, R. A.; Chandraratna, R. A. S. *Fundam. Appl. Toxicol.* **1996**, *33*, 264.
7. Soprano, D. R.; Soprano, K. J. *Annu. Rev. Nutr.* **1995**, *15*, 111.
8. (a) Zusi, F. C.; Reczek, P. R.; Ostrowski, J. U.S. Patent 6,008,251, 1999; . *Chem. Abstr.* **1998**, *132*, 35518.

9. Oh, Gi-Su.; Pae, Hyun-Ock.; Seo, Won-Gil.; Shin, Min-Kyo.; Kim, Il-Kwang.; Chai, Kyu-Yun.; Yun, Young-Gab.; Kwon, Tae-Oh.; Lim, Joung-Sik.; Chung, Hun-Taeg. *Immunopharmacol. Immunotoxicol.* **2001**, *23*, 335.
10. Datta, P. K.; Lianos, E. A. *Kidney Int.* **1999**, *56*, 486.
11. Kim, B. H.; Kang, K. S.; Lee, Y. S. *Toxicol. Lett.* **2004**, *150*, 191.
12. Manfredini, S.; Simoni, D.; Caminiti, G.; Vertuani, S.; Invidiata, F.; Moscato, B.; Hatse, S.; De Clercq, E.; Balzarini *J. Med. Chem. Res.* **1998**, *8*, 291.
13. Garattini, E.; Parrella, E.; Diomede, L.; Gianni, M.; Kalac, Y.; Merlini, L.; Simoni, D.; Zanier, R.; Ferrara, F. F.; Chiarucci, I.; Carminati, P.; Terao, M.; Pisano, C. *Blood* **2004**, *103*, 194.
14. Simoni, D.; Grisolia, G.; Giannini, G.; Roberti, M.; Rondanin, R.; Piccagli, L.; Baruchello, R.; Rossi, M.; Romagnoli, R.; Invidiata, F. P.; Grimaudo, S.; Jung, M. K.; Hamel, E.; Gebbia, N.; Crosta, O. L.; Abbadessa, O. V.; Di Cristina, A.; Dusonchet, L.; Meli, M.; Tolomeo, M. *J. Med. Chem.* **2005**, *48*, 723.
15. Anzaldi, M.; Sottofattori, E.; Rizzetto, R.; Granello Di Casaleto, B.; Balbi, A. *Eur. J. Med. Chem.* **1999**, *34*, 837.
16. Dallegri, F.; Ottonello, L.; Dapino, P.; Sacchetti, C. *J. Rheumatol.* **1992**, *19*, 419.
17. Dallegri, F.; Dapino, P.; Arduino, N.; Bertolotto, M.; Ottonello, L. *Antimicrob. Agents Chemother.* **1999**, *43*, 2307.
18. Dallegri, F.; Ottonello, L.; Ballestrero, A.; Bogliolo, F.; Ferrando, F.; Patrone, F. *Gut* **1990**, *31*, 184.
19. Dallegri, F.; Ottonello, L. *Inflamm. Res.* **1997**, *46*, 382.
20. Sottofattori, E.; Grandi, T.; Balbi, A. *Tetrahedron Lett.* **1995**, *36*, 1331.
21. Hensby, C. N.; Eustache, J.; Shroot, B.; Bouclier, M.; Chatelus, A.; Luginbuhl, B. *Agents Actions* **1987**, *21*, 238.
22. Duberttret, L.; Lebreton, C.; Touraine, R. *Br. J. Dermatol.* **1982**, *107*, 681.
23. Hensby, C.; Cavey, M.; Bouclier, A.; D'algate, C.; Eustache, J.; Shroot, B. *Agents Actions* **1990**, *29*, 1.
24. Shalita, A. *J. Eur. Acad. Dermatol. Venereol.* **2001**, *15*, 43.
25. Bershad, S. V. *Mt. Sinai J. Med.* **2001**, *68*, 279.
26. Ottonello, L.; Dapino, P.; Amelotti, M.; Barbera, P.; Arduino, N.; Bertolotto, M.; Dallegri, F. *Inflamm. Res.* **1998**, *47*, 345.
27. Sottofattori, E.; Anzaldi, M.; Balbi, A. *J. Heterocycl. Chem.* **1998**, *35*, 1377.
28. Ottonello, L.; Dapino, P.; Scirocco, M. C.; Balbi, A.; Bevilacqua, M.; Dallegri, F. *Clin. Sci.* **1995**, *88*, 331.
29. Dankberg, F.; Persidsky, M. D. *Cryobiology* **1976**, *13*, 430.
30. Leslie, R. G. *J. Immunol. Methods* **1987**, *103*, 253.
31. Ottonello, L.; Gnerre, P.; Bertolotto, M.; Mancini, M.; Dapino, P.; Russo, R.; Garibotto, G.; Barreca, T.; Dallegri, F. *J. Am. Soc. Nephrol.* **2004**, *15*, 2366.