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Synthesis and immunomodulatory properties of selected oxazolone derivatives

Muhammad A. Mesaik,[a] Shagufta Rahat,[b] Khalid M. Khan,[b,*] Zia-Ullah,[b] Muhammad I. Choudhary,[b] Shahnaz Murad,[c] Zakiah Ismail,[c] Atta-ur-Rahman[b] and Aqeel Ahmad[a,*]

[a]*Department of Microbiology, University of Karachi, Karachi 75270, Pakistan*
[b]*HEJ Research Institute of Chemistry, International Center for Chemical Sciences, University of Karachi, Karachi 75270, Pakistan*
[c]*Institute for Medical Research Kuala Lumpur, 50588 Kuala Lumpur, Malaysia*

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Abstract—Eleven oxazolone derivatives were synthesized and characterized by ^{1}H NMR, EI, IR and UV spectroscopic and CHN analysis. Three compounds, 4-[(*E*)-(4-nitrophenyl)methylidene]-2-phenyl-1,3-oxazol-5(4*H*)-one (**11**), 4-[(*E*)-(4-methoxyphenyl)-methylidene]-2-methyl-1,3-oxazol-5-one (**12**) and 4-[(*E*)-(4-nitrophenyl)methylidene]-2-methyl-1,3-oxazol-5(4*H*)-one (**13**) were screened for phagocyte chemiluminescence, neutrophil chemotaxis, T-cell proliferation, cytokine production from mononuclear cells and cytotoxicity. 4-[(*E*)-(4-Nitrophenyl)methylidene]-2-methyl-1,3-oxazol-5(4*H*)-one (**13**) was found to be the most potent immunomodulator in the series.

1. Introduction

The knowledge of the inter-related and multiple levels of interactions between various components of the immune system have helped in understanding how the immune system provides protection against the foreign invaders and pathological conditions. This has also led to the development of many drugs that modulate the host natural defense mechanism and restore impaired immune function.[1] Immunomodulation denotes to any change in the immune response and may involve induction, expression, amplification or inhibition of any part or phase in the immune response. Stimulation of the immune response is required in certain patients, whereas, suppression of the immune response is needed in other conditions.[2] Novel immunomodulating agents are used for the treatment of various conditions such as infections, organ transplantation, cancer, rheumatoid arthritis, systemic lupus erythematosus, etc.[3–5]

The immunomodulatory drugs such as cyclosporin isolated from a soil fungus *Trichoderma polysprm*[6] and tacrolimus (FK-506), a secondary metabolite of *Streptomyces tsukabaensis*, are used to suppress immunological rejection of the transplanted organs.[7] These unmodified natural products represent major breakthroughs in organ transplantation. Based on these natural products and their activities, new immunomodulator drugs were synthesized, which are among the most important anticancer drugs used today. Tamoxifen, an antiestrogenic compound and methotrexate, which is a folic acid antagonist, are two examples of synthetic drugs that inhibits DNA synthesis and cell division.[8–10]

Oxazolone is a class of small heterocycles, which are important intermediates in the synthesis of several small molecules, including amino acids, peptides,[11–16] antimicrobial or antitumour compounds,[17,18] heterocyclic precursors[19–22] as well as biosensors coupling and or photosensitive composition devices for proteins.[23] Some oxazolones have shown a wide range of pharmaceutical properties.[24]

In continuation of our drug discovery program,[25–29] we synthesized a variety of oxazolones and screened their effects on different aspects of immune response. The

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* Corresponding authors. Tel./fax: +92-21-4989018 (A.A.); fax: +92-21-9243190-91 (K.M.K.); e-mail addresses: hassaan2@super.net.pk; aahmad57@hotmail.com

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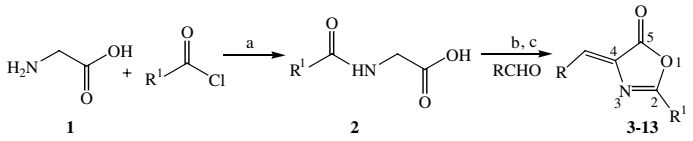


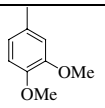
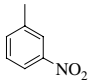
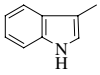
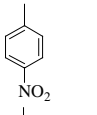
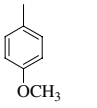
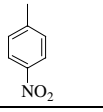
Scheme 1. Reagents and conditions: (a) BzCl, or AcCl, 10% NaOH, H_2O; (b) Ac_2O, NaOAc, reflux; (c) PPA, 80–90 °C.

compounds were tested for phagocyte chemiluminescence, neutrophil chemotaxis, T-cell proliferation, cytokine production from mononuclear cells and cytotoxicity.

2. Results

2.1. Chemistry

The compounds **3**–**13** were synthesized from commercially available glycine with acetic anhydride/benzoyl chloride in the presence of anhydrous sodium acetate followed by Erlenmeyer condition with different aldehydes in very high yields.[30–32] All the oxazolones were isomerized by heating them with polyphosphoric acid on a water bath 80–90 °C for 2 h according to literature procedure.[33] The structures were determined by using different spectroscopic methods like ^{1}H NMR, EI, IR and UV and confirmed by CHN analysis (Scheme 1).

Compound	R	R^1
3		
4		
5		
6		
7	Isobutyl	
8		
9		
10		
11		
12		$-CH_3$
13		$-CH_3$

2.2. Biological activity

In order to test the immunomodulating effects of the test compounds, we used a number of assays in different immune-responsive cells. We investigated oxidative burst activity of neutrophils and monocytes, chemotoxis in neutrophils, T-cell proliferation and cytokine release by lymphocytes. We used prednisolone a known immune suppressive drug as a positive control in these studies. We initially tested 11 oxazolones derivatives for their possible immunomodulatory activities using inhibition of zymosan-induced phagocytosis. Three compounds **11**, **12** and **13** showed varying degrees of suppressive activities (96%, 32% and 78%, respectively), whereas rest of the compounds **3**–**10** either showed moderate or no activities (Table 1). We, therefore, used compounds **11**, **12** and **13** for further evaluation and the results are summarized as follows.

2.2.1. Effect on MDBK cell proliferation. The test compounds were tested for possible cellular toxic effects by determining cell proliferation on Mavin Darby Bovine Kidney (MDBK) cells (Table 2). Results presented in Table 1 indicate that the test compounds as high as 50 μg/mL did not exhibit any toxic effect after 3 days incubation with MDBK cells. Therefore various doses up to 50 μg/mL were used during present investigation.

2.2.2. Effect on phagocytes oxidative burst. Phagocytic cells on activation induce release of reactive oxygen free

Table 1. Effect of compounds on oxidative burst (chemiluminescence assay)

Compound	50 μg/mL	25 μg/mL
3	24	19
4	2	12
5	11	17
6	14	10
7	35	9
8	10	0
9	46	12
10	32	7
11	85	78
12	93	32
13	99	96

Two concentrations of compounds **3**–**13** were incubated with neutrophil for 30 min. After the addition of serum treated zymosan (STZ) and luminol, neutrophils were scanned at 37 °C for their CL activity for 50 min. Compounds activity was compared with the control. The percentage effect was calculated as = 100 − {(CL in presence of compound/CL count in the absence of compound) × 100}. The reading represents a triplicate result of each concentration.

Table 2. Compounds IC_{50} value from the MTT cytotoxicity studies

Compound	IC_{50} (μg/mL)	
	5 days MTT results	3 days MTT results
11	6.04	⩾50.0
12	20.6	Nil
13	6.0	Nil

Serial compounds dilution (0.195–50 μg/mL) were incubated with Mavin Darby bovine kidney (MDBK) cells for 72 h and cells viability was evaluated by MTT reduction to the blue coloured formazan in living cells. All values were means of 3–5 replicate. Nil = no cytotoxicity detected.

radicals (oxidative burst), which is then quantified by a luminol-enhanced chemiluminescence assay. Results presented in Figure 1 indicate that the zymosan-induced oxidative burst in polymorphoneutrophils (PMNs) was inhibited potently by compound **13** at a concentration of 1.56 μg/mL. However, compound **13** was less active in suppressing the activity of the monocytes, 20% inhibition was obtained at 3.125 μg/mL. Compound **12** was found to be less active than compounds **11** and **13**. The inhibition of the oxidative burst was not due to cytotoxic effects as none of these compounds were found to exhibit cytotoxicity (Table 2).

2.2.3. Effect on polymorphoneutrophil cells chemotoxis. Effect of the test compounds on PMN chemotoxis in response to a chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP 10^{-8}) was assayed using Boyden chambers. PMNs were incubated with 25 and 50 μg/mL of each compound for 1 h and the difference in the cell movement was studied. Prednisolone was run as a positive control. The data presented in Figure 2 show that all tested compounds exhibited chemotoxis suppressive activity. Compounds **11** and **12** at 25 μg/mL

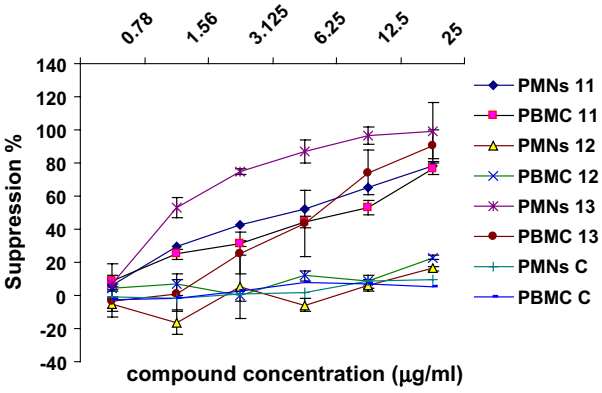


Figure 1. Effect of compounds on oxidative burst by phagocyte cells. Various concentrations of compounds **11**, **12** and **13** were incubated with polymorphoneutrophil (PMNs) or peripheral blood mononuclear cells (PBMC) for 30 min. After the addition of serum treated zymosan (STZ) and luminol, phagocytic cells were scanned at 37 °C for their chemiluminescence (CL) activity. The compounds activity was compared with the control (C). The percentage effect was calculated as = 100 − {(CL in presence of compound/CL count in the absence of compound) × 100}. Each plot and error bar represents reading ± SD of three repeats.

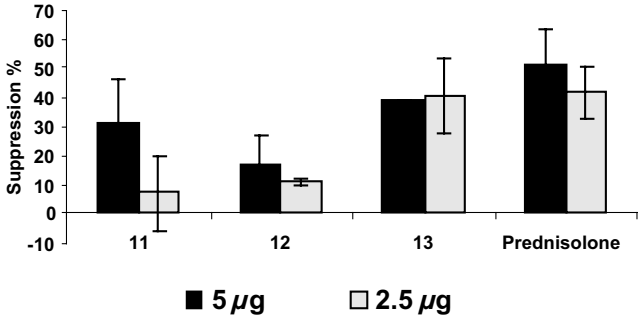


Figure 2. Effect of test compounds on chemotoxis. Effect of the test compounds on the PMNs chemotoxis was evaluated using Boyden chamber (Neuro Probe, USA). The chemoattractant (fMLP 10^{-8}) was added into the lower chamber. Compounds and cells were introduced simultaneously into the upper chamber at 37 °C. The movement of cells in the filter membranes was observed under the light microscope. fMLP = formyl-methionyl-leucyl-phenylalanine. Each plot and error bar represents reading ± SD of three repeats.

inhibited only 7–12% fMLP-induced chemotoxis, whereas at 50 μg/mL the inhibition of 32% and 17%, was observed, respectively. Compound **13** exhibited the most potent suppressive activity on fMLP-induced PMNs movement and caused about 40% inhibition of chemotoxis at 25 μg/mL concentration, which did not change significantly at 50 μg/mL concentration.

2.2.4. Effect on T-cell proliferation. The anti-proliferation effect of the test compounds was determined by measuring the inhibition of phytohemagglutinin (PHA)-induced T-cell proliferation by determining radioactive thymidine incorporation. Results shown in Figure 3 indicate that all three test compounds significantly suppressed T-cell proliferation in dose dependent manner. However, the compounds **11** and **13** showed the

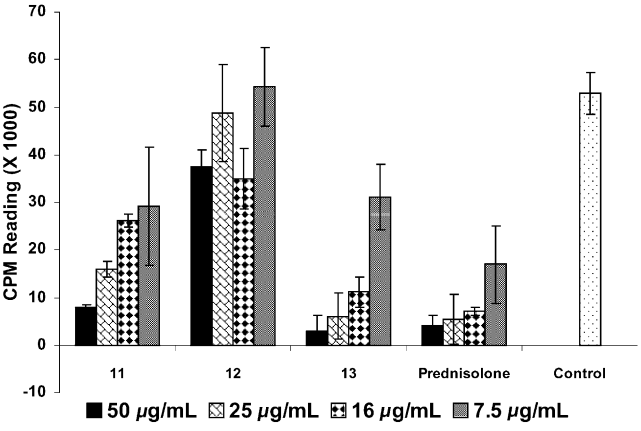


Figure 3. Effect of compounds on phytohemagglutinin (PHA) T-cell proliferation. Cells were incubated with different concentrations of the test compounds in RPMI-1640 along with PHA for 72 h at 37 °C in CO_2 environment. Cells were further incubated for 18 h after the addition of thymidine [^{3}H] and the radioactivity count as CPM reading was recorded using beta scintillation counter. The effect of compounds on the T-cell proliferation is compared with control. Each bar represents the mean value of triplicate reading ± SD.

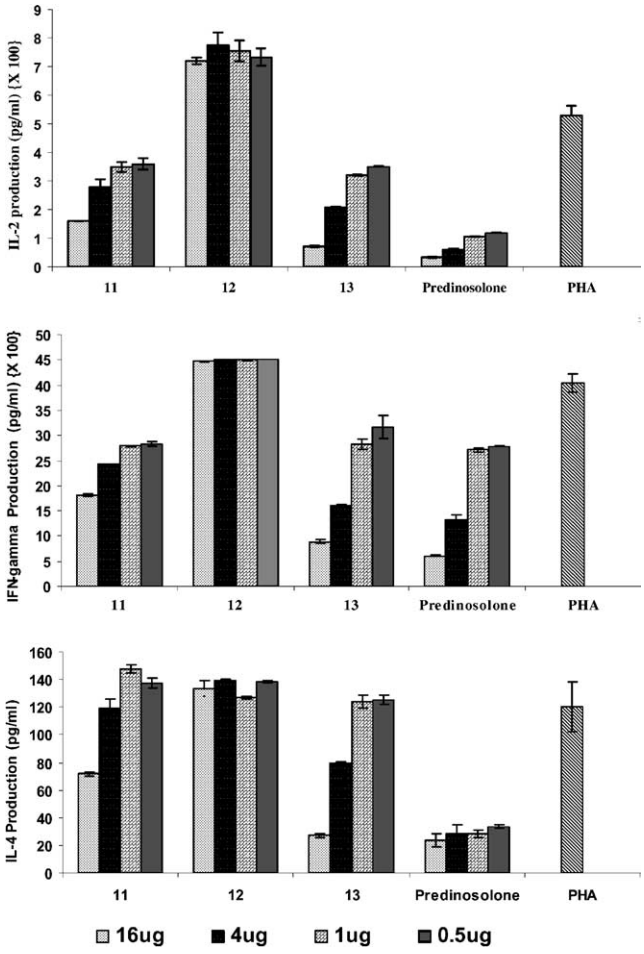


Figure 4. Effect of the test compounds on cytokine production. Four concentrations (0.5, 1, 4 and 16 μg/mL) from each compound were incubated with the peripheral blood mononuclear cells (PBMC) cells for 18 h along with the stimulant (PHA). Level of IL-2, INF-γ and IL-4 was determined by cytokine ELISA kits (Diaclone, Besancon Cedex, France) compare to control (PHA). Each bar represents the mean value of triplicate reading ± SD level. IL-2 level = 528.5 ± 34.52, INF-γ level = 4033.7 ± 180, IL-4 level = 120.5 ± 18.1.

most significant suppressive activity. A dose as low as 7.5 μg/mL of compounds **11** and **13**, caused approximately 40–50% reduction in T-cell proliferation compared to control. The anti-proliferation effect of these compounds was further increased with concentration and the cell proliferation was reduced by 75–85% at 50 μg/mL. Compound **12** had least potent anti-proliferation activity and only 10–15% inhibition was observed with 16–50 μg/mL concentrations.

2.2.5. Effect on cytokine release by peripheral blood mononuclear cells (PBMC). The suppressive effect of the test compounds was tested on the release of selected cytokine including IL-2, IFN-γ and IL-4 by PHA-induced lymphocytes. Results shown in Figure 4 indicate that compounds **11** and **13** apparently inhibited IL-2, IL-4 and INF-γ release in a dose dependent manner from PHA-activated lymphocytes. A dose as low as 0.5 μg/mL was found to be very effective for these compounds. In contrast compound **12** has no effect on the inhibition of any cytokine release from activated lymphocytes.

3. Statistics

All data are presented as means ± standard deviation of the mean. Statistical analysis for all the results was compared using student's *t*-test. Significance was attributed to probability values $P \leqslant 0.05$ ($P \leqslant 0.005$ in some cases). The IC_{50} values were calculated using Excel based program.

4. Discussion

A number of assays were used to investigate the immunomodulating effect of oxazolone derivatives on different immune cells. Early in the phagocytosis process, neutrophils undergo a respiratory burst, generating free radicals, which react with luminol and emit light.[34] Thus, chemiluminescence enhanced by the presence of luminol may be used as an indicator of phagocytic activity.[35] The present data show that compounds exert inhibitory effect on phagocytic activity of PMNs and PBMC. It was also found to interfere with the phytohemagglutinin (PHA) T-cell activation in a dose dependant manner. The impaired incorporation of tritiated thymidine into cellular DNA may not be due to toxic effect since the compound was found to be non-toxic against MDBK cell line with a similar incubation time. The effect of compound **11** on T-cell proliferation was further confirmed by suppression of IL-2 and IFN-γ production. These two compounds **11** and **13** are clearly exerting immunomodulatory activity on the system used and could be suitable immunomodulatory lead compounds for future research.

The results of preliminary assays and structures of these compounds indicated that oxazolones with a suitably substituted exocyclic phenyl ring possess immunosuppressive activity. The possible structural factor, which influences the activity, is exocyclic substitution on oxazolone at C-4. The compounds having exocyclic *p*-substituted nitrophenyl rings showed varying degrees of activities as in case of compounds **3**, **11**, **12** and **13**. The most active compound of the series was **13**, which fulfil essential structural requirements, described above.

Furthermore weak activities were observed in case of compounds **12** and **3** (Table 1). However, both compounds contain *para*-methoxy group on exocyclic phenyl ring. The activity difference between compounds **12** and **3** might be due to the presence of an additional methoxy group at *meta*-position of compound **3**, which possibly restrict the molecule to interact with cells.

In case of compounds **11**, **12** and **13**, which were active at the preliminary chemiluminescence (CL) screening, a

detailed study of these compounds was carried out, which includes phagocyte chemiluminescence, neutrophils chemotaxis, T-cell proliferation assay, cytokine production from mononuclear cells and cytotoxicity evaluation.

The chemotoxis studies were carried out on compounds, **11**, **12** and **13**. The compound **13** was found most active (38.6%) at a 50 μg/mL concentration. However, at the lower concentration (25 μg/mL) level, it showed slight increase (40%) in the inhibition. The other tested compounds **11** and **12** showed weak activity in this assay at both concentrations (Fig. 2). The results suggested that methyl chain and exocyclic substituted phenyl group might participate in interaction of these compounds with phagocytic cells movement.

In case of T-cell activation, the percentage inhibition of compounds **11** and **13** showed that compound **13** was more potent at all concentrations (Fig. 3). The results also suggested that the activity might be due to the presence of a methyl and exocyclic nitro substituted phenyl group.

The result on IL-2, IFN-γ and IL-4 release suggests that compounds **13** and **11** were the most potent compounds, whereas compound **12** was not suppressing the production of any of the tested cytokine, rather a slight increase is observed on IL-2 production (Fig. 4). Effect of compound **13** again can be attributed to methyl group and exocyclic nitro substituted phenyl group. The IL-4 studies on the aforementioned compounds showed that compound **13** is the most potent in series (Fig. 4), which might be because of the earlier described reasons.

5. Conclusions

The data presented above indicate that a C-4 *para*-nitro substituted exocyclic phenyl group on oxazolone moiety greatly influences the immunosuppressive activity. The nitro substitution at *para*-position contributes in activity, while a methoxy group at the same position decreases the activity. These studies also suggest that *para*-nitro substituted exocyclic phenyl ring at C-4 might enhance the interaction of the molecule with cell. However, further mechanism-based studies are required for a better understanding of the mechanism of action of oxazolones on immune response.

6. Experimental section

6.1. General

Melting points were determined with a Büchi SMP-20 apparatus and are uncorrected. The ultraviolet spectra were measured in chloroform on a Lambda 5 UV/vis spectrometer (Perkin–Elmer). IR spectra (KBr discs) were recorded on a Brüker FT-IR IFS 48 spectrometer. EI and FD mass spectral data were recorded with Varian MAT 711 (70 eV) spectrometer, and data are tabulated as m/z. Elemental analyses were carried out on a Perkin Elmer 2400 CHN Elemental Analyzer. ^{1}H NMR spectra were recorded, using tetramethylsilane as an internal standard, on a Brüker AC 250 (300 and 400 MHz) spectrometer, respectively. Chemical shifts are reported in δ (ppm) and coupling constants are given in Hz. The progress of all reactions was monitored by TLC on 2×5 cm pre-coated plates with silica gel 60 F_{254} to a thickness of 0.25 mm (Merck). The chromatograms were visualized under ultraviolet light (254–366 nm) and iodine vapours.

6.1.1. *N*-Benzoylglycine (2a). Glycine (10 g, 133.0 mmol) was dissolved in 10% sodium hydroxide solution (100 mL), and then benzoyl chloride (21.6 mL, 186.0 mmol) was added in portions to this solution, stirred vigorously after each addition. Crushed ice (100 g) was added to the solution and then concentrated HCl was added dropwise until the mixture was acidified (pH 2–3). The resulting compound **2** was obtained as white crystalline solid 23.0 g (96%); ^{1}H NMR (300 MHz, CD_3OD) δ: 4.09 (s, 2H, CH_2), 7.46 (d, 2H, $J = 8.1$ Hz, Ar-H), 7.52 (d, 1H, $J = 7.2$ Hz, Ar-H), 7.85 (dd, 2H, $J = 1.5$, 8.4 Hz, Ar-H), 8.17 (br s, 1H, NH); IR (KBr): 3457, 3029, 1771, 1721; UV λ_{max} ($CHCl_3$): 268, 209 nm; EI-MS m/z (rel abund %): 179 (M^+, 29), 134 (100), 102 (59), 77 (83). Anal. Calcd for $C_9H_9NO_3$: C, 60.33; H, 5.06; N, 7.82. Found: C, 60.39; H, 5.00; N, 7.85.

6.1.2. *N*-Acetylglycine (2b). A solution of glycine (10 g, 133.0 mmol) in water (135 mL) was dissolved, then acetic anhydride (24 mL, 266.0 mmol) was added to it in one portion and vigorously stirred for 30 min, cooled overnight, solid was collected and washed with ice cold water, dried to afford compound **2b** as white solid 7.14 g (46%). Mp: 208 °C; ^{1}H NMR (300 MHz, CD_3OD) δ: 1.99 (s, 3H, CH_3), 3.89 (s, 2H, CH_2), 8.22 (br s, 1H, NH); IR (KBr): 3461, 1765, 1732; UV λ_{max} ($CHCl_3$): 209 nm; EI-MS m/z (rel abund %):117 (M^+, 8), 82 (100), 100 (78). Anal. Calcd for $C_4H_7NO_3$: C, 41.03; H, 6.03; N, 11.96. Found: C, 41.00; H, 6.06; N, 11.99.

6.1.3. 4-[(*E*)-(3,4-Dimethoxyphenyl)methylidene]-2-phenyl-1,3-oxazol-5(4*H*)-one (3). A solution of 3,4-dimethoxy benzaldehyde (1.50 g, 9.0 mmol), *N*-benzoyl- glycine (2.0 g, 11.0 mmol), acetic anhydride (3.0 mL, 33.6 mmol), fused sodium acetate (1.0 g, 12.1 mmol) was heated, after liquification, heating was continued for additional 2 h. After the completion of reaction (TLC analysis) 4.4 mL ethanol was added and kept at room temperature for 18 h. It was filtered, solid thus obtained was isomerized by mixing and then heating with polyphosphoric acid on a steam bath 80–90 °C for 2 h and then poured into water.[33] The resultant solid product was collected, washed with cold ethanol, hot water, ethanol and then small amount of hexane and then dried to afford compound **3** as pale yellow solid; Yield: 1.96 g (70%). Mp: 149.3 °C; R_f: 0.87 (*n*-butanol–acetic acid–H_2O 3:3:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ:

3.92 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3), 7.19 (br s, 1H, H-6), 6.91 (s, 1H, H-6′), 7.40 (t, 1H, $J = 7.2$ Hz, H-4″), 7.50 (d, 1H, $J = 7.5$ Hz, H-6′), 7.57 (d, 1H, $J = 7.2$ Hz, H-5′), 8.10 (d, 2H, $J = 6.9$ Hz, H-2″,6″), 8.15 (dd, 2H, $J = 1.8$, $J = 6.9$ Hz, H-3″,5″); IR (KBr): 1783, 1771, 1666, 1497, 1462, 1329, 1286, 1160, 1112, 1013, 994, 917, 889, 867 cm^{-1}; UV λ_{max} ($CHCl_3$): 396, 260, 201 nm; EI-MS m/z (rel abund %): 309 (M^+, 43), 176 (6) 131 (2), 105 (100), 51 (5). Anal. Calcd for $C_{18}H_{15}NO_4$: C, 69.89; H, 4.89; N, 4.52. Found C, 69.87; H, 4.88; N, 4.50.

6.1.4. 4-[(*E*)-(3-Nitrophenyl)methylidene]-2-phenyl-1,3-oxazol-5(4*H*)-one (4). The compound **4** was prepared in the same way as described for compound **3** from 3-nitro benzaldehyde (1.20 g, 7.94 mmol). The product was obtained as a light orange solid 1.55 g (66%). Mp: 162 °C; R_f: 0.64 (*n*-butanol–acetic acid–H_2O 3:3:1); ^1H NMR (300 MHz, $CDCl_3$) δ: 7.24 (br s, 1H, H-6), 7.54 (t, 1H, $J = 8.2$ Hz, H-3′), 7.59–7.65 (m, 1H, H-4″, Ar-H), 7.67 (dd, 2H, $J = 3.8$, $J = 8.0$ Hz, H-3″,5″), 8.18 (dd, 2H, $J = 1.3$, $J = 8.0$ Hz, H-2″,6″), 8.28 (dd, 1H, $J = 1.3$, $J = 8.2$ Hz, H-2″), 8.40 (d, 1H, $J = 8.2$ Hz, H-4′), 9.30 (s, 1H, H-6′, Ar-H); IR (KBr): 1788, 1657, 158, 1349, 1294, 1163, 994, 889 cm^{-1}; UV λ_{max} ($CHCl_3$): 3576, 268, 225 nm; EI-MS m/z (rel abund %): 294 (M^+, 46), 105 (100), 77 (50). Anal. Calcd for $C_{16}H_{10}N_2O_4$: C, 65.30; H, 3.43; N, 9.52. Found: C, 65.28; H, 3.42; N, 9.58.

6.1.5. 4-[(*E*)-1*H*-Indol-3-ylmethylidene]-2-phenyl-1,3-oxazol-5(4*H*)-one (5). Indole-3-carbaldehyde (1.50 g, 10.34 mmol), *N*-benzoylglycine (2.5 g, 13.97 mmol), acetic anhydride (3.75 mL) and fused sodium acetate were treated in the same way as described for compound **3**. The product was obtained as yellow solid 1.81 g (61%). Mp: 206 °C; R_f: 0.65 (*n*-butanol–acetic acid–H_2O 3:3:1); ^1H NMR (300 MHz, $CDCl_3$) δ: 7.49 (s, 1H, H-6), 7.42 (dd, 2H, $J = 1.7$, $J = 7.4$ Hz, H-3″,5″), 7.54 (t, 2H, $J = 7.4$ Hz, H-2″,6″), 7.62 (t, 1H, $J = 7.4$ Hz, H-4″), 7.84 (dd, 1H, $J = 6.8$, $J = 7.8$ Hz, H-5′), 8.13 (d, 2H, $J = 7.2$ Hz, H-2, H-6′,7′), 8.47 (d, 1H, $J = 7.4$ Hz, H-8′), 8.75 (s, 1H, CH); IR (KBr): 1781, 1646, 1581, 1339, 1291, 1153, 987, 875 cm^{-1}; UV λ_{max} ($CHCl_3$): 400, 377, 296, 203, 194 nm; EI-MS m/z (rel abund %): 288 (M^+, 51), 105 (100), 77 (34). Anal. Calcd for $C_{18}H_{12}N_2O_2$: C, 74.99; H, 4.20; N, 9.72. Found: C, 74.89; H, 4.25; N, 9.82.

6.1.6. 4-[(*E*)-(3-Chlorophenyl)methylidene]-2-phenyl-1,3-oxazol-5(4*H*)-one (6). 3-Chlorobenzaldehyde (1.98 g, 14.1 mmol), *N*-benzoylglycine (2.5 g, 13.97 mmol), acetic anhydride (3.93 mL, 41.7 mmol) and fused sodium acetate (1.14 g, 13.9 mmol) were treated in the same way as described for compound **3**. The product was obtained as yellow solid 1.58 g (40%). Mp: 166 °C; R_f: 0.67 (*n*-butanol–acetic acid–H_2O 3:3:1); ^1H NMR (400 MHz, $CDCl_3$) δ: 7.16 (s, 1H, H-6), 7.40–7.42 (m, 3H, H-3″,4″,5″, Ar-H), 7.52 (dd, 1H, $J = 0.93$, $J = 8.2$ Hz, H-2′, H-3′), 7.56 (d, 2H, $J = 6.4$ Hz, H-2″,6″), 7.61 (d, 1H, $J = 8.2$ Hz, H-2″), 8.18 (dd, 1H, $J = 2.2$, $J = 7.1$ Hz, H-4′, Ar-H), 8.28 (s, 1H, H-6′, Ar-H); IR (KBr): 1786, 1641, 1563, 1486, 1388, 1268, 1184, 1066, 982, 743 cm^{-1}; UV λ_{max} ($CHCl_3$): 378, 372, 359, 259, 255, 251, 243, 230, 203, 198 nm; EI-MS m/z (rel abund %): 283 (M^+, 63), 141 (9), 104 (100), 51 (39). Anal. Calcd for $C_{16}H_{10}NO_2$: C, 67.74; H, 3.55; N, 4.94. Found: C, 67.73; H, 3.54; N, 4.96.

6.1.7. 4-[(*E*)-3-Methylbutylidene]-2-phenyl-1,3-oxazol-5(4*H*)-one (7). Isovaleraldehyde (1.63 mL, 15.3 mmol), *N*-benzoylglycine (2.5 g, 13.96 mmol), acetic anhydride (3.93 mL, 36.3 mmol) and fused sodium acetate (1.14 g, 13.9 mmol) were treated in the same way as described for compound **3**. The orange yellow product obtained was 2.0 g (63%). Mp: 136 °C; R_f: 0.56 (*n*-butanol–acetic acid–H_2O 3:3:1); ^1H NMR (300 MHz, $CDCl_3$) δ: 1.50 (d, 6H, $J = 7.5$ Hz, $(CH_3)_2$), 2.95 (m, 1H, CH), 7.24 (s, 1H, H-6), 7.35 (d, 2H, $J = 7.9$ Hz, H-3′,5′), 7.46 (t, 1H, $J = 7.6$ Hz, H-4′), 7.54 (d, 2H, $J = 7.9$ Hz, H-2′,6′); UV λ_{max} ($CHCl_3$): 382, 378, 274, 237, 202, 194 nm; EI-MS m/z (rel abund %): 229 (M^+, 45), 105 (100), 77 (45). Anal. Calcd for $C_{14}H_{15}NO_2$: C, 73.34; H, 6.60; N, 6.10. Found: C, 73.33; H, 6.59; N, 6.11.

6.1.8. 4-[(*E*)-Pyrrol-3-ylmethylidene]-2-phenyl-1,3-oxazol-5(4*H*)-one (8). Compound **8** was prepared in the same way as described for compound **3**, from pyrrole carbaldehyde (1.5 g, 15.77 mmol). The product was obtained as yellow solid 2.26 g (60%); R_f: 0.66 (*n*-butanol–acetic acid–H_2O 3:3:1); ^1H NMR (300 MHz, $CDCl_3$) δ: 7.19 (br s, 1H, H-6), 7.3–7.8 (m, 3H, H-2′,3′,4′), 8.5–8.8 (m, 5H, H-2″,3″,4″,5″,6″, Ar-H); FD MS m/z: 238 (M^+); IR (KBr): 1795, 1775, 1665; 1555, 1515, 1480, 1460, 1410, 1380, 1360, 1315, 1245, 1125, 1095, 965, 875, 865, 765 cm^{-1}; UV λ_{max} ($CHCl_3$): 374, 273, 227, 262, 262 nm. Anal. Calcd for $C_{14}H_{10}N_2O_2$: C, 70.58; H, 4.23; N, 11.76. Found: C, 70.55; H, 4.22; N, 11.77.

6.1.9. 4-[(*E*)-(3-Aminophenyl)methylidene]-2-phenyl-1,3-oxazol-5(4*H*)-one (9). 3-Amino benzaldehyde (1.85 g, 15.3 mmol), *N*-benzoylglycine (2.5 g, 13.9 mmol), acetic anhydride (3.93 mL, 36.3 mmol) and fused sodium acetate (1.14 g, 13.89 mmol) were treated in the same way as described for compound **3**. The yellow solid product obtained was 1.85 g (50%). Mp: 179 °C; R_f: 0.65 (*n*-butanol–acetic acid–H_2O 3:3:1); ^1H NMR (300 MHz, $CDCl_3$) δ: 4.24 (s, 2H, NH_2), 7.24 (br s, 1H, H-6), 7.4 (t, 1H, $J = 7.8$ Hz, H-2′), 7.51 (t, 1H, $J = 7.8$ Hz, H-3′), 7.60 (d, 1H, $J = 7.2$ Hz, H-4′), 7.69 (d, 2H, $J = 8.0$ Hz, H-2″,6″), 7.70 (d, 2H, $J = 8.0$ Hz, H-3″), 8.17 (t, 1H, $J = 7.2$ Hz, H-4″), 8.30 (s, 1H, H-6′); EI-MS m/z (rel abund %): 264 (M^+, 13), 248 (39), 171 (56), 127 (63), 105 (100), 77 (83); IR (KBr): 3458, 1769, 1751, 1655, 1576, 1486, 1367, 1278, 1163, 1061, 980, 739 cm^{-1}; UV λ_{max} ($CHCl_3$): 313, 269, 227, 215, 200, 197, 193 nm. Anal. Calcd for $C_{16}H_{12}N_2O_2$: C, 72.72; H, 4.58; N, 10.60. Found: C, 72.72; H, 4.57; N, 10.60.

6.1.10. 4-[(*E*)-(2-Bromophenyl)methylidene]-2-phenyl-1,3-oxazol-5(4*H*)-one (10). 2-Bromobenzaldehyde (1.79 mL, 15.29 mmol), *N*-benzoylglycine (2.5 g, 13.96 mmol), acetic anhydride (3.93 mL, 36.3 mmol) and fused sodium

acetate (1.25 g, 15.23 mmol) were treated in the same way as described for compound **3**. The product was obtained as yellow solid 2.54 g (55%). Mp: 144 °C; R_f: 0.55 (*n*-butanol–acetic acid–H_2O 3:3:1); ^1H NMR (400 MHz, $CDCl_3$) δ: 7.13 (br s, 1H, H-6), 7.26 (d, 1H, $J = 7.4$ Hz, H-2′), 7.44 (t, 1H, $J = 7.4$ Hz, H-3′), 7.52 (t, 1H, $J = 7.4$ Hz, H-4′), 7.60 (d, 1H, $J = 7.4$ Hz, H-5′), 8.16 (d, 2H, $J = 7.6$ Hz, H-2″,6″), 8.87 (d, 2H, $J = 7.5$ Hz, H-3″,5″), 8.37 (s, 1H, H-6′); EI-MS m/z (rel abund %): 330 (M+2, 19), 328 (M^+, 20), 105 (100), 77 (54); IR (KBr): 3127, 1807, 1763, 1673, 644; UV ($CHCl_3$): 311, 262, 231, 211, 201, 197, 194 nm. Anal. Calcd for $C_{16}H_{10}BrNO_2$: C, 58.56; H, 3.07; N, 4.27. Found: C, 58.59; H, 3.04, N, 4.29.

6.1.11. 4-[(*E*)-(4-Nitrophenyl)methylidene]-2-phenyl-1,3-oxazol-5(4*H*)-one (11). Compound **11** was prepared in the same way as described for compound **3**, from 4-nitrobenzaldehyde (2.31 g, 15.28 mmol), *N*-benzoylglycine (2.0 g, 11.1 mmol), acetic anhydride (3.0 mL, 33.6 mmol), fused sodium acetate (1.0 g, 12.1 mmol). The product was obtained as yellow solid 2.0 g (61%). R_f: 0.68 (*n*-butanol–acetic acid–H_2O 3:3:1); ^1H NMR (300 MHz, $CDCl_3$) δ: 7.24 (br s, 1H, H-6), 7.40–7.42 (m, 1H, H-4″, Ar-H), 7.50 (d, 2H, $J = 6.0$ Hz, H-3″,5″), 7.57 (d, 2H, $J = 6.0$ Hz, H-2″,6″), 8.01 (dd, 2H, $J = 1.5$, $J = 7.8$ Hz, H-3′,5′), 8.08 (dd, 2H, $J = 2.1$, $J = 7.8$ Hz, H-2′,6′); IR (KBr): 1790, 1765, 1665, 1590, 1555, 1465, 1355, 1150, 975, 860 cm^{-1}; UV λ_{max} ($CHCl_3$): 423, 282, 261 nm; EI-MS m/z (rel abund %): 294 (M^+, 45), 105 (100), 77 (75). Anal. Calcd for $C_{16}H_{10}N_2O_4$: C, 65.30; H, 3.43; N, 9.52. Found: C, 65.28; H, 3.45; N, 9.50.

6.1.12. 4-[(*E*)-(4-Methoxyphenyl)methylidene]-2-methyl-1,3-oxazol-5(4*H*)-one (12). Compound **12** was prepared in the same way as described for compound **3** from 4-methoxy benzaldehyde (1.20 g, 12.28 mmol), *N*-acetylglycine (2.0 g, 11.1 mmol), acetic anhydride (3.0 mL, 33.6 mmol) and fused sodium acetate (1.0 g, 12.1 mmol). The product was obtained as light orange solid 2.0 g (64%). Mp: 158 °C; R_f: 0.66 (*n*-butanol–acetic acid–H_2O 3:3:1); ^1H NMR (300 MHz, $CDCl_3$) δ: 2.18 (s, 3H, CH_3), 3.88 (s, 3H, OCH_3), 7.0 (d, 2H, $J = 8.8$ Hz, H-3′,5′), 7.22 (s, 1H, H-6), 8.17 (t, 2H, $J = 8.8$ Hz, H-2′,6′); IR (KBr): 1793, 1764, 1651, 1314, 1306, 1166, 984, 896 cm^{-1}; UV λ_{max} ($CHCl_3$): 385, 259, 201 nm; EI-MS m/z (rel abund %): 279 (M^+, 27), 105 (100) 51 (6). Anal. Calcd for $C_{17}H_{13}NO_3$: C, 73.10; H, 4.70; N, 5.01. Found: C, 73.11; H, 4.69; N, 5.00.

6.1.13. 4-[(*E*)-(4-Nitrophenyl)methylidene]-2-methyl-1,3-oxazol-5(4*H*)-one (13). Compound **13** was prepared in the same way as described for compound **3**, from 4-nitrobenzaldehyde (2.31 g, 15.28 mmol), *N*-acetylglycine (2.0 g, 11.1 mmol), acetic anhydride (3.0 mL, 33.6 mmol), fused sodium acetate (1.0 g, 12.1 mmol). The product was obtained as yellow solid 2.0 g (61%). R_f: 0.68 (*n*-butanol–acetic acid–H_2O 3:3:1); ^1H NMR (300 MHz, $CDCl_3$) δ: 2.18 (s, 3H, CH_3), 7.24 (br s, 1H, H-6), 8.01 (dd, 2H, $J = 1.5$, $J = 7.8$ Hz, H-3′,5′), 8.08 (dd, 2H, $J = 2.1$, $J = 7.8$ Hz, H-2′,6′); IR (KBr): 1783, 1757, 1666, 1587, 1561, 1468, 1356, 1152, 974, 866 cm^{-1}; UV λ_{max} ($CHCl_3$): 423, 282, 261 nm; EI-MS m/z (rel abund %): 295 (M^+, 44.97), 105 (100), 77 (75.68). Anal Calcd for $C_{16}H_{10}N_2O_4$: C, 65.30; H, 3.43; N, 9.52. Found C, 65.28; H, 3.45; N, 9.50.

6.2. Phagocyte chemiluminescence

Luminol-enhanced chemiluminescence assay was performed using Helfand et al. protocol.[36] Briefly 5×10^5 neutrophils, suspended in modified Hank's solution (MHS), were incubated with varying concentrations of compounds (0.78–25 μg/mL) for 30 min. Zymosan (Sigma Chemical Co., USA) 100 μL (20 mg/mL), followed by 100 μL (7×10^5 M) luminol (Sigma Chemical Co., USA) was added to make a final volume of 0.25 mL. MHS alone was run as a control. Peak chemiluminescence was recorded with the luminometer (Labsystem Luminoskan RS, Finland). The luminometer was set with repeated scan mode, 50 scans with 30 s intervals and one second point measuring time.

6.3. Neutrophil chemotaxis

Chemotaxis was measured using a modified Boyden chamber (Neuro Probe, Cabin John, MD, USA), as previously described.[37] Briefly to the lower compartment 25 μL of the formyl-methionyl-leucyl-phenylalanine (fMLP) (10^{-8} M) chemoattractant (Sigma Chemical Co., USA) was added. The upper compartment was separated from the lower compartment by a cellulose filter (2 μm pore size; Poretics Co., USA). To the upper compartment 5×10^4 cells in a 50 μL HBSS (with calcium and magnesium) was added. Compounds (25 and 50 μg/mL) and control were added each in duplicate following the addition of cells. The chemoattractant buffer (DMSO and HBSS without calcium and magnesium, 1:1 ratio) was added as a control. The chamber was incubated at 37 °C in CO_2 environment for 60 min. Cells within the filter were stained with hematoxylin and their travelling distance was measured with the help of light microscope in five randomly chosen fields per filter.

6.4. T-Cell proliferation assay

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood of healthy adult donor by Ficoll–Hypaque gradient centrifugation.[38] Cells were proliferated following a method reported by Nielsen et al.[39] Briefly cells were cultured at a concentration of 5×10^5/mL in a 96 well round bottom tissue culture plate (Nalge Nunc. Inter.). Cells were stimulated with 1.25 μg/mL of phytohemagglutinin (Sigma Co., USA). Various concentrations of oxazolone compounds were added to give final concentrations of 7.5, 16, 25 and 50 μg/mL each in triplicate. The plate was incubated for 72 h at 37 °C in 5% CO_2 incubator. After 72 h, cells were

pulsed with (0.5 μCi/well) tritiated thymidine, and further incubated for 18 h. Cells were harvested onto a glass fibre filter (Cambridge Technology, USA) using cell harvester (SKATRON A.S. Flow Lab. Norway). The tritiated thymidine incorporation into the cells was measured by a liquid scintillation counter (1211 LKB WALLAC). CPM results were recorded after 120 s.

6.5. Cytokine production from mononuclear cells

Cytokine from mononuclear cells was assayed using the human cytokine kits (Diaclone, Besancon Cedex, France). Briefly, freshly prepared mononuclear cells (10^5/well) were cultured in 96-well microtiter plate in the presence or absence of 1.25 μg/mL PHA. Four different concentrations (0.5, 1, 4 and 16 μg/mL) of each compound along with PHA were used in this assay. Plate was incubated at 37 °C in CO_2 environment. After 18 h of incubation, the supernatant was collected and analyzed for IL-2, IL-4 and IFN-γ cytokine production.

6.6. Cytotoxicity evaluation

The MTT test was performed to evaluate the cytotoxicity effect of the compounds according to the following method described previously.[40] Briefly MTT was dissolved in 1× PBS at 2 mg/mL. Mavin Darby bovine kidney (MDBK) adherent cell (2×10^5) were incubated with serial concentrations of compounds (0.195–50 μg/mL) for three/five days. Supernatant was removed and 50 μL of MTT solution was then added. Plates were incubated for an additional 2–3 h at 37 °C in a tissue culture incubator. MTT was aspirated off and 200 μL of DMSO was added to dissolve the formazan crystal formed by living cells. The plate was agitated at room temperature for 15 min then read at 540 nm using microplate reader.

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