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Synthesis of New Pyrrolo[1,2-*d*][1,2,4]triazines and Thiazolo[3,4-*d*][1,2,4]triazines as Immunostimulating Agents

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Abstract—Four pyrrolo[1,2-*d*][1,2,4]triazines and four thiazolo[3,4-*d*][1,2,4]triazines were synthesized from *trans*-4-hydroxy-L-proline and L-thiaproline, respectively. The synthetic route involved formation of hydrazides followed by cyclization with orthoesters. The proliferative response to human lymphocyte mitogen (phytohemagglutinin) revealed significant immunostimulant activity for all test drugs. Furthermore, some triazine derivatives were effective to activate production of free oxygen radical by phagocytes in response to stimulation by opsonized zymosan.

Introduction

The clinical need for therapeutic agents able to restore a normal immune response in immunocompromised patients has led to the discovery of a number of substances collectively defined as immunomodulators.[1,2] Stimulation of the nonspecific immune response may be expected to significantly assist patients affected with infectious diseases and cancers. Presently, whereas natural macromolecular immunostimulants (bacterial, extracts, cytokines, grow factors) are being increasingly used in human medicine, only a small number of synthetic immunostimulants are efficient in clinical practice.[3] Microbial products and cytokines tend to be pleiotropic, influencing several parts of functions of the immune system. In contrast chemical immunomodulators could theoretically be prepared with selective effects acting at specific points in the immune response. One promising issue is to discover compounds acting on T lymphocytes either by enhancing T cell mediated activation or by favoring T cell differentiation.[4,6] The first synthetic compound acting by stimulation of T cell function in vivo was an imidazothiazole derivative, the levogyre enantiomeric form of tetramisole (i.e., levamisole).[7,8] This one is efficacious in immunodeficient individuals presumably through an action of its sulphur moiety to induce a thymic hormone-like factor.[1] The toxicity of levamisole, however, reduced the maximum prescribed dose of this agent giving rise to the development of a research program for the synthesis of chemically-related compounds.[5,8–12] In view of the immunoregulatory actions on human T lymphocytes of previously synthesised pyrrolopyrimidinediones and thiazolohydantoins,[13,14] we decided to extend our investigations to some structurally related derivatives. We prepared a series of pyrrolo and thiazolotriazines in order to evaluate the effect on activity of replacing the pyrimidine or the hydantoin ring by a triazine nucleus, while maintaining the bridgehead atom present in the aforementioned immunoagents.

Chemistry

The effects of modification of the diazabicyclooctane ring system on biological potency were examined through two series of compounds prepared as described in Scheme 1. Esters **1a** and **1b** were obtained from *trans*-4-hydroxy-L-proline and L-thiaproline, respectively, according to previously reported methods.[15,16] The action of hydrazine hydrate on **1** furnished hydrazides **2**

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in good yields. Cyclization of **2** with an appropriate orthoester afforted the target pyrrolotriazines **3** and thiazolotriazines **4**. Stereochemistry of compounds **3** and **4** was established from NOE experiments. Irradiation of the H_7 proton caused an increase of the intensity of both the $H_{6\beta}$ and $H_{8\beta}$ protons of **3**. In addition irradiation of $H_{8\alpha}$ concomitantly caused an increase of $H_{8\beta}$, H_{8a}, and OH. At last when $H_{8\beta}$ was irradiated, NOE was observed at the signals of H_7 and $H_{8\alpha}$. Based on the foregoing evidence, the stereochemistry of triazines **3** was concluded to be 7*R* and 8a*S* (Scheme 1). Similar NOE effects were observed in the case of thiazolo derivatives **4** supporting a configuration *R* for the carbon 8a.

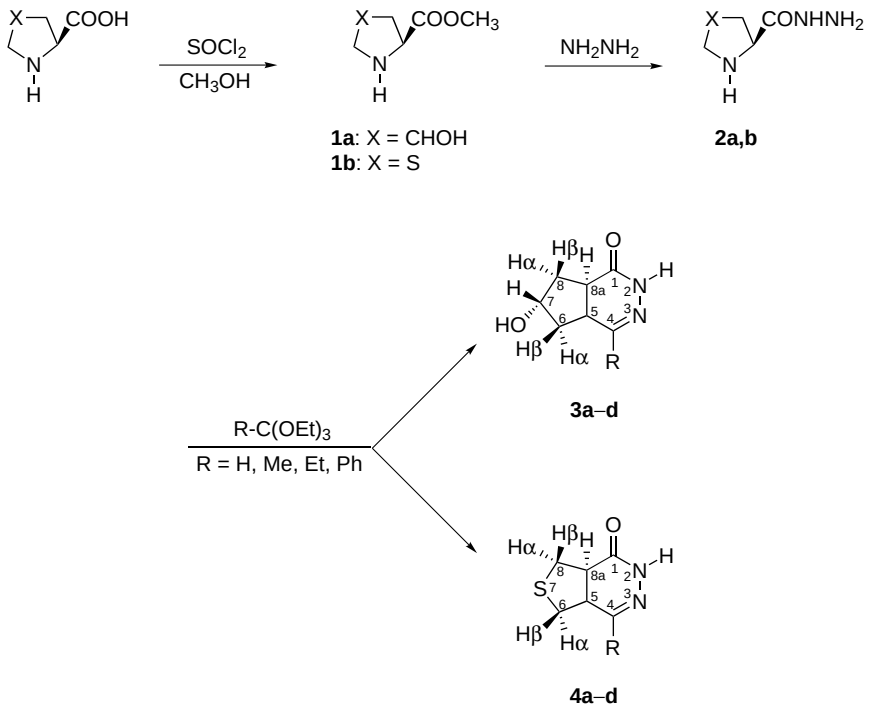
Physical constants and spectral data of **3** and **4** are reported in Tables 1 and 2, respectively. For ^{13}C NMR, all spectra of bicyclic compounds showed a signal about 162 ppm attributable to the C=0 group.

Biological Results

The biological activity of the triazine derivatives **3** and **4** was first determined by the ability of these compounds to stimulate the proliferative response of lymphocytes to a mitogen.[17] In a second time, compounds **3** and **4** were tested for immunostimulant activity by checking the effects on free oxygen radical (FOR) production by phagocytes in response to stimulation by opsonized yeast stimulus (zymosan).[18–20]

Proliferative response: MTT assay

Briefly, a mitogenic stimulation assay using T cell mitogen phytohemagglutinin (PHA) was performed with peripheral blood leukocytes taken from healthy donors. Proliferating cells were evaluated using a quantitative colorimetric assay with 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). This colorimetric assay converts yellow tetrazolium salt to its blue formazan derivative via the mitochondrial enzymes in viable cells. Assays for compounds and for levamisole, used as reference, were performed on leukocytes from ten blood donors. The responses to PHA are shown in Figure 1. Proliferative activity induced by levamisole, was significantly increased in all concentrations tested when compared to that of control ($\mathbf{p} = 0.004$). Similarly all compounds, whatever the concentrations used, had significant positive effects on the proliferation of lymphocytes when compared to control ($p < 0.05$). In addition several compounds significantly (*) increased proliferation as compared to levamisole ($p < 0.05$). These effects were particularly remarkable for the following derivatives: **3a**, **4a**, **4b** at 50 μg/mL and **3a**, **3b**, **4a** at 100 μg/mL. At the concentrations used (between 1



Scheme 1.

and 100 μg/mL), the stimulating effects on the proliferative response varied only slightly ($p > 0.05$). None of the compounds activities was dose-dependent.

Chemiluminescence assay

Assays for compounds and Biostim®, used as the reference, were performed on blood phagocytes from nine donors. The oxidative response measured by evaluating FOR production and induced only by zymosan (control) or by zymosan and compounds is shown in Figure 2. Only the peak value of CL at the concentration with the greatest oxidative response is shown as the median.

FOR production induced by Biostim® was significantly increased in all concentrations tested when compared to that of control ($p < 0.001$). Except for **4a**, all compounds increased FOR production as compared to control. But only three compounds had significant effects: **3b** at 100 μg/mL ($p = 0.009$), **4b** at 10 μg/mL ($p = 0.02$), and **4d** at 10 μg/mL ($p = 0.01$). No compound had greater effect than Biostim® on FOR production.

Discussion

Results on the proliferative response of blood lymphocytes showed that test drugs **3** and **4** had greater immunostimulating activity than did the reference drug, levamisole at the same doses. They all provided potent stimulation indexes in this model with minimum effective doses of 1 μg/mL. Although derivatives **3** represented an attempt to assess the contribution to activity of the sulphur atom of the thiazolotriazines, there was no significant difference between the two chemical series. Moreover, the nature of the substituent in the 4-position

Table 1. Physical constants of pyrrolo-triazines **3a–d** and thiazolo-triazines **4a–d**

Compound	R	Formula	M.W.	Yield (%)	mp ± 1 (°C)
3a	H	$C_6H_9O_2N_3$	155.15	33	191
3b	CH_3	$C_7H_{11}O_2N_3$	169.18	54	186
3c	C_2H_5	$C_8H_{13}O_2N_3$	183.21	25	182
3d	C_6H_5	$C_{12}H_{13}O_2N_3$	231.25	12	226
4a	H	$C_5H_7ON_3S$	157.19	15	126
4b	CH_3	$C_6H_9ON_3S$	171.21	54	130
4c	C_2H_5	$C_7H_{11}ON_3S$	185.24	33	117
4d	C_6H_5	$C_{11}H_{11}ON_3S$	233.28	9	135

Table 2. Spectral data of pyrrolo-triazines **3a–d** and thiazolo-triazines **4a–d**

Compound	IR ν (cm^{-1}) C=O; C=N	^{1}H NMR (DMSO-d_6)-δ (ppm)
3a	1665; 1635	1.85 (1H, ddd, J=13, 11, 5 Hz, C_8-H), 2.0 (1H, dd, J=13, 6 Hz, C_8-H), 3.2 (1H, dd, J=11, 2 Hz, C_6-H), 3.75 (1H, dd, J=11, 5 Hz, C_6-H), 4.0 (1H, dd, J=11, 6 Hz, C_{8a}-H), 4.3 (1H, m, C_7-H), 5.1 (1H, s, OH), 7.0 (1H, s, C_4-H), 10.2 (1H, s, NH)
3b	1670; 1625	1.85 (1H, m, C_8-H), 1.9 (3H, s, CH_3), 2.0 (1H, dd, J=13, 6 Hz, C_8-H), 3.2 (1H, d, J=11 Hz, C_6-H), 3.8 (1H, dd, J=11, 5 Hz, C_6-H), 3.95 (1H, dd, J=11, 6 Hz, C_{8a}-H), 4.3 (1H, m, C_7-H), 5.1 (1H, s, OH), 10.1 (1H, s, NH)
3c	1660; 1610	1.1 (3H, t, J=7 Hz, CH_3), 1.9 (1H, ddd, J=13, 11, 5 Hz, C_8-H), 2.0 (1H, dd, J=13, 6 Hz, C_8-H), 2.2 (2H, m, CH_2), 3.2 (1H, d, J=11 Hz, C_6-H), 3.8 (1H, dd, J=11, 5 Hz, C_6-H), 3.95 (1H, dd, J=11, 6 Hz, C_{8a}-H), 4.3 (1H, m, C_7-H), 5.1 (1H, s, OH), 10.1 (1H, s, NH)
3d	1665; 1610	2.2 (2H, m, C_8-H), 3.3–3.5 (2H, m, C_6-H), 4.2 (2H, m, C_{8a}-H and C_7-H), 5.2 (1H, s, OH), 7.4–7.6 (5H, m, C_6H_5), 10.6 (1H, s, NH)
4a	1660; 1630	2.8 (1H, t, J=10 Hz, C_8-H), 3.4 (1H, dd, J=10, 7 Hz, C_8-H), 3.9 (1H, dd, J=10, 7 Hz, C_{8a}-H), 4.3 (1H, d, J=9 Hz, C_6-H), 4.9 (1H, d, J=9 Hz, C_6-H), 7.0 (1H, s, C_4-H), 10.7 (1H, s, NH)
4b	1670; 1635	1.95 (3H, s, CH_3), 2.9 (1H, t, J=10 Hz, C_8-H), 3.4 (1H, dd, J=10, 7 Hz, C_8-H), 3.8 (1H, dd, J=10, 7 Hz, C_{8a}-H), 4.2 (1H, d, J=9 Hz, C_6-H), 4.9 (1H, d, J=9 Hz, C_6-H), 10.5 (1H, s, NH)
4c	1670; 1630	1.0 (3H, t, J=7Hz, CH_3), 2.3 (2H, m, CH_2), 2.8 (1H, t, J=10 Hz, C_8-H), 3.3 (1H, dd, J=10, 7 Hz, C_8-H), 3.9 (1H, dd, J=10, 7 Hz, C_{8a}-H), 4.2 (1H, d, J=9 Hz, C_6-H), 4.9 (1H, d, J=9 Hz, C_6-H), 10.5 (1H, s, NH)
4d	1665; 1610	3.4 (2H, m, C_8-H), 4.2 (1H, m, C_{8a}-H), 4.45 (1H, d, J=9 Hz, C_6-H), 4.55 (1H, d, J=9 Hz, C_6-H), 7.5 (5H, m, C_6H_5), 11.0 (1H, s, NH)

of the triazine ring did not significantly influence biological effects; therefore compounds **3c** and **4c** were not tested.

In contrast, results for the oxidative response of blood phagocytes demonstrated a difference between the two series of derivatives. In the case of pyrrolotriazines, **3b** with a methyl group induced a significant increase when compared to the activity of the unsubstituted analogue **3a** and to that of **3d**, possessing a phenyl nucleus. Considering thiazolotriazine, methyl compound **4b** and phenyl derivative **4d** increased significantly the production of FOR. Surprisingly, **4a** differing from the parent compound **4b** only in its lack of substituent on the triazine ring, exhibited a markedly different effect since it induced a decrease of FOR production when compared to control.

More precise interpretation of the structure activity relationships is difficult on account of the limited number of molecules tested. In previously reported works,[13,14] it was postulated that a dioxo-group as well as two five-membered rings in the bicyclic system were necessary to improve the immunomodulating activity. In view of the last data, it appears that the bicyclic skeleton tolerates much more variations than we

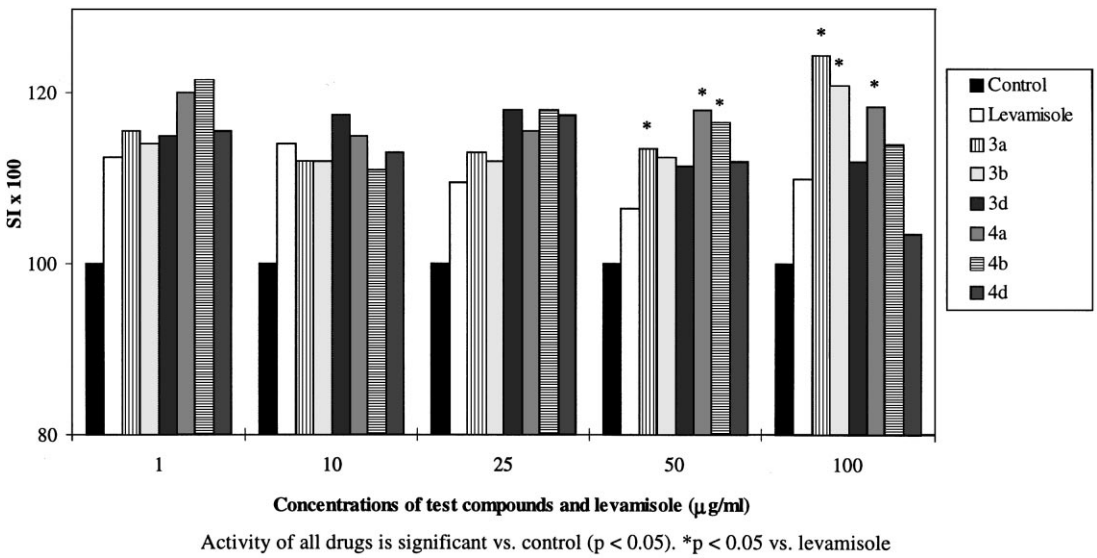


Figure 1. Proliferative response: stimulation index (SI) of human lymphocytes stimulated by PHA and incubated with and without (control) compounds (median values of 10 assays).

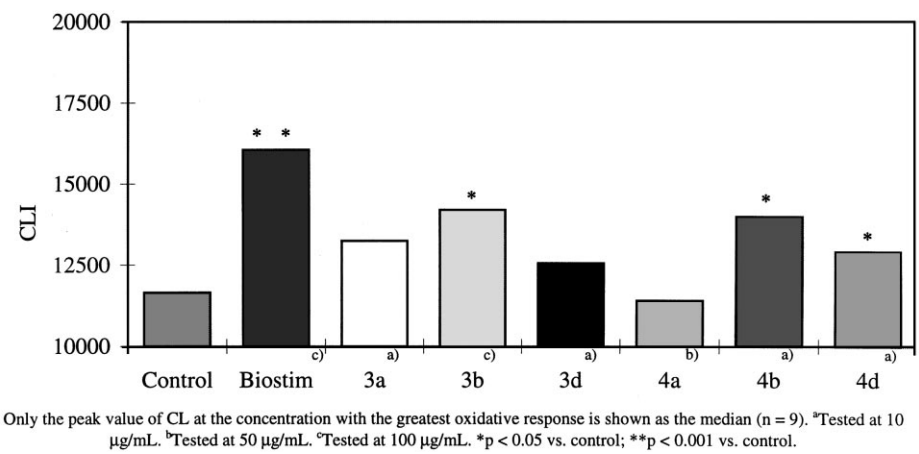


Figure 2. Oxidative response: chemiluminescence index (CLI) of human phagocytes stimulated by opsonized zymosan and induced with and without (control) compounds.

supposed, which justified the synthesis and the investigation of additional series to specify the structural features contributing to potent immunostimulating activity.

None of the compounds activities was dose-dependent for the two tests. However, it is possible to state that the two series of compounds were immunostimulants for the proliferation of lymphocytes and for FOR production of phagocytes whatever dose used. This result is particularly attractive, considering that in closely related chemical series it was observed simultaneous immunosuppressive and immunostimulating properties according to concentration of products.[9,11] Due to their potent activity in the two tests used, triazinones **3b** and **4b** appeared as the most promising compounds for further pharmacological and toxicological studies. In addition to the uncertainty relative to in vitro studies, in vivo experiments are required to confirm interest of these derivatives. Obviously the in vivo activity will be a reflection not only of the inherent activity of the compounds but pharmacokinetic/pharmacodynamic parameters as well.

Experimental

Chemistry

Melting points were determined on a Kofler apparatus without correction. Infrared (IR) spectra were recorded on a Beckman 4240 spectrophotometer in KBr disks. All ^{1}H and ^{13}C spectra were recorded on a Bruker AC 400 (400 MHz) spectrophotometer using tetramethylsilane as an internal standard. The coupling constants are in Hertz (Hz) and the chemical shifts are reported in parts per million (δ, ppm). The following abbreviations are used : s = singlet, d = doublet, t = triplet, b = band, m = multiplet, br s = broad singlet. Elemental analyses were performed at the Service Central d'Analyses, Centre National de la Recherche Scientifique, 69390 Vernaison, France. Analytical results obtained for all compounds were within ±0.4% of theoretical values. For analytical TLC, plastic sheets coated with a 0.2 mm layer of silica-gel 60 F_{254} (Merck) were used and spots were visualized with UV light. When required, the separation of crude reaction products was achieved by chromatography on a silica gel column (35–70μ).

2-Hydrazido-4-hydroxy-pyrrolidine (2a). To a solution of **1a** (2.47 g, 17 mmol), in ethanol (10 mL) was added hydrazine hydrate (1 g, 20 mmol) and the resulting solution was stirred at room temperature during 18 h. After evaporation, the crude product was recrystallized in ethanol to provide 2 g (81%) of **2a**; mp 139 °C. IR (KBr) 3300, 2900, 1650, 1550 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.7 (1H, ddd, J = 13, 8.5, 5 Hz, C_3-H), 1.95 (1H, dd, J = 13, 8 Hz, C_3-H), 2.7 (1H, d, J = 12 Hz, C_5-H), 2.9 (1H, dd, J = 12, 4 Hz, C_5-H), 3.7 (1H, t, J = 8 Hz, C_2-H), 3.7–4.1 (4H, b, OH, NH, NH_2), 4.2 (1H, m, C_4-H), 8.9 (1H, br *s*, N*H*-NH_2).

4-Hydrazido-thiazolidine (2b). A mixture of **1b** (2.5 g, 17 mmol), hydrazine hydrate (1 g, 20 mmol) and ethanol (10 mL) was stirred for 24 h and then evaporated. The crude material was chilled in an ice bath. The resulting crystals were collected and purified by recrystallization from hexane to afford 2.17 g (87%) of **2b**; bp 77°C. IR (KBr) 3280, 1650 cm^{-1}; ^{1}H NMR (DMSO-d_6) d 2.8 (1H, dd, J = 10, 7 Hz, C_5-H), 2.95 (1H, dd, J = 10, 7 Hz, C_5-H), 3.15 (1H, br s, NH), 3.65 (1H, t, J = 7Hz, C_4-H), 4.0 (1H, d, J = 9 Hz, C_2-H), 4.3 (1H, d, J = 9 Hz, C_2-H), 4.25 (2H, b, NH_2), 9.2 (1H, s, N*H*-NH_2).

7-Hydroxy-4-methyl-1-oxo-6,7,8,8a-tetrahydro-2*H*-pyrrolo[1,2-*d*][1,2,4]triazine (3a). To a solution of **2a** (1.02 g, 7 mmol) in DMF (10 mL) was added triethyl orthoformiate (1.25 g, 7.7 mmol). The mixture was refluxed for 8 h and evaporated to dryness. The resulting solid was recrystallized in methanol to obtain **3a** as white plates.

Compounds **3b**, **3c**, and **3d** were prepared similarly to **3a** with appropriate triethyl orthoesters, but derivatives **3c** and **3d** were purified by column chromatography (eluent:ethylacetate:methanol, 9:1).

1-Oxo-5,6,8,8a-tetrahydro-2*H*-thiazolo[3,4-*d*][1,2,4]triazines (4). Compounds **4** were prepared in the same manner as described for **3**, using **2b** as starting material and heating for 6 h. They were purified by column chromatography with ethyl acetate as eluent.

Immunopharmacology

Proliferative response to mitogenic stimulation (PHA): MTT assay. Peripheral blood leukocytes from healthy donors were separated by centrifugation on a ficoll-hypaque gradient. Cells were cultured in RPMI 1640 (Gibco BRL, Cergy Pontoise, France) containing 10% foetal calf serum (DAP, Volgebrun, France). The cell suspension was diluted to 10^6 cells/mL, and 100 μL put into each well of the 96-well plates. Phytohemagglutinin (PHA) was used at final concentration of 25 μL/mL. Incubation was carried out with and without (without = control) potential immunomodulating compounds at various concentrations (1, 10, 25, 50, and 100 μg/mL), for 48 h. All cells were counted using the blue trypan (0.25%) exclusion test, and viability was always over 95%. A stock solution of MTT (Sigma, St Quentin, France) was dissolved in saline phosphate buffer at 5 mg/mL and sterilized by filtering. 100 μL was

added to each well, and plates were incubated at 37 °C for 3 h. Plates were then centrifuged and dimethylsulfoxyde was added to all wells. After a few minutes at room temperature, the plates were read with a Titertek Multiskan Plus (a microplate reader) using a test wavelength of 570 nm, and a reference wavelength of 630 nm. Results calculated from triplicates, were expressed as a stimulation index (SI) using the following formula: SI = OD (T cells stimulated by PHA + compound)/O.D (T cells stimulated by PHA), (OD: optic density). For control, the value of SI is 100%, indicating the absence of proliferative response. Below this value, results indicate immunosuppressing activity, and above immunostimulating activity.

Oxidative response: chemiluminescence assay. Peripheral blood, containing phagocytes, was directly transferred to tubes and diluted at 1/10 in Hanks for basal measurements. We then added appropriately diluted stimulating agents : opsonised zymosan-A from *Saccharomyces cerevisiae* (20 mg/mL) (Sigma Chemical, St. Louis, MO, USA) for the control, and opsonised zymosan-A and compounds at different concentrations. All were incubated with 50 µL of luminol (5-amino-2,3-dihydro-1,4-phtalazine-dione) (Boehringer Mannheim, Germany) for 30 min. The kinetic measurement of chemiluminescence, performed every 2 min by a biolumat LB 9500 C photometer (Berthold, Wilbad, Germany) was expressed in Relative Light Units (RLU). For chemiluminescence response (CL) evaluation, we established a chemiluminescence index (CLI) as follows: CLI = RLU (stimulated cells)−RLU (unstimulated cells)/RLU (unstimulated cells). Only the peaks of CL were utilized. Biostim® (RU41740) was used as reference, and kindly supplied by Cassenne–Roussel UCLAF Laboratories (Paris, France). RU41740 was dissolved in proportions of 5 mg/mL lyophilized powder in sterile saline solution.

Statistical analysis. Because of the small sample size, results were expressed as median values. Statistical comparisons were made using the non-parametric Mann–Whitney *U* test.[21]

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