

5,6-Dihydro-2*H*-pyranones and 5,6-dihydro-2*H*-pyridones and their derivatives modulate in vitro human T lymphocyte function

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Received: 9 June 2011 / Accepted: 13 August 2011 / Published online: 30 August 2011
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Abstract The aim of this work was to study the in vitro effects of δ -lactone **1**, δ -lactam **3** and their enaminone derivatives **2** and **4**, synthesized in our laboratory, on the proliferative responses of human lymphocytes, Th1 and Th2 cytokine secretion and intracellular redox status. Peripheral blood lymphocytes were isolated using differential centrifugation on a density gradient of Histopaque. They were cultured with mitogen concanavalin A (Con A) and with different concentrations of the compounds **1**, **2**, **3** and **4** (0.1–10 μ M). Proliferation (MTT assay), IL-2, INF γ and IL-4 (Elisa kits), oxidative markers (intracellular glutathione, hydroperoxide and carbonyl protein contents) and cytotoxic effect (micronucleus test) were determined. The compounds **1** and **2** are immunosuppressive and decrease IL-2, INF γ and IL-4 secretion with a shift away from Th2 response to Th1 phenotype. The compounds **3** and **4** were immunostimulant and increased cytokine secretion with a

shift away from Th1 response to Th2. The introduction of an enamine group to **1** and **3** to provide **2** and **4** seemed to attenuate their immunological properties. These immunomodulatory properties were, however, accompanied by an increase in lymphocyte intracellular oxidative stress, especially with **1** and **2** at high concentrations. In conclusion, the compounds **1**, **2**, **3** and **4** could be used to provide cell-mediated immune responses for novel therapies in T-cell mediated immune disorders.

Keywords Cytokines · δ -Lactams · δ -Lactones · Human lymphocytes · In vitro proliferation · Oxidative stress

Introduction

To improve bioavailability for medical applications, a number of 5,6-dihydro-2*H*-pyranones or δ -lactones and 5,6-dihydro-2*H*-pyridones or δ -lactams compounds have been synthesized and characterized for more than a decade. These compounds have been extensively studied for their structural features and biological functions. They are produced from plants and fungi or by chemical synthesis and display potent antitumor, anti-invasive, cardiovascular and neurotropic activities [1–5]. They have been shown to possess bactericidal properties, plant stimulatory activity [6, 7] and inhibit some enzymes [8–10]. It has been widely accepted that δ -lactones and δ -lactams have immunomodulating properties, including regulation of cell differentiation and effector functions of different immune cells and modulation of cytokine production [11, 12]. In particular, they have an inhibitory effect on lymphocyte proliferation and downregulate interleukin (IL)-2 and TNF- α

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secretion and, in consequence, the activation of Th1 lymphocytes in humans [13, 14].

The normal function of the immune system is essential for health, and dysfunction of the immune system leads to several diseases. The most relevant cells involved in the immune response are lymphocytes, but other cells are also implicated, including monocytes/macrophages and granulocytes [15]. The majority of immune diseases are linked to a loss of T-cell homeostasis. The healthy immune system is held in balanced equilibrium, apparently by the contra-suppressive production of cytokines by T-helper 1 (Th1) and T-helper 2 (Th2) lymphocyte subsets. Cytokines are involved in signaling between cells during an immune response. Interleukins are an important group of cytokines mainly produced by T-cells. IL-2, an important cytokine produced by Th1 lymphocytes, supports the continuous exponential growth of human T-cells and also acts as a differentiation molecule that promotes T-cytotoxic cell activity and B-cell activity [16]. IFN γ is another important Th1-cytokine that enhances NK cell activity, induces the generation of T cytotoxic cells, activates macrophages for tumor killing and antimicrobial activity and modulates the expression of class II major histocompatibility complex (MHC) molecules. Th2 cells secrete IL-4 which is involved in the modulation of antibody production and in the suppression of cell-mediated immunity and inflammation [17].

The proliferation, the activation and the cytokine secretion by T-cells are regulated by the formation of intracellular reactive oxygen species (ROS) suggesting that intracellular ROS could play a role in peripheral T-cell homeostasis [18, 19]. The metabolism of hydroperoxides through the glutathione (GSH) represents one of the major cellular defense mechanisms against oxidative stress [20]. In the absence of oxidative stress, 90–95% of GSH is in its reduced state [21]. In response to a stress, intracellular GSH is consumed by forming GSH conjugates or by forming GSSG. Depletion of GSH in human T-cells was shown to impair IL-2 production, which is known to stimulate T-cell proliferation [22].

Insufficient information is currently available about the effects of δ -lactones and δ -lactams on lymphocyte function including proliferation, cytokine production and intracellular redox status although antitumor effects have been previously reported [1, 23]. To explore these immune properties, we synthesized δ -lactones and δ -lactams and their enaminone derivatives according to a mechanism described previously [24–26] and modified in our laboratory. δ -Lactone **1** and δ -lactam **3** were obtained by the condensation of 4-hydroxy-4-methyl-2-pentanone with cyanoacetic ester and cyanoacetamide in the presence of ammonium acetate [24, 26]. The synthesis of their enaminolactone **2** and enaminolactam **4** was carried out by

reacting **1** and **3** with *N,N*-dimethylformamide dimethyl acetal as previously reported by Baldwin et al. [25]. Therefore, in this study, we investigated the in vitro effects of compounds **1**, **2**, **3** and **4** on mitogen-stimulated proliferation, Th1- and Th2-type cytokine production and oxidant/antioxidant status of human lymphocytes. The in vitro micronucleus test was used to test if these compounds induce DNA damage. Our initial work constitutes a first step to explore the immunomodulation of these molecules.

Materials and methods

Chemical synthesis

All chemicals used in this study were purchased from Sigma-Aldrich.

Synthesis of **1** and **3**

A mixture of β cetol 4-hydroxy-4-methyl-2-pentanone (0.01 mol), cyanoacetic ester (0.01 mol) and acetate ammonium (0.005 mol) was heated under reflux for 6 h at 80°C (Fig. 1a). The precipitate was collected, filtered and washed with diethyl ether to provide the δ -lactone, 4,6,6-trimethyl-2-oxo-5,6-dihydro-2*H*-pyran-3-carbonitrile **1**. To prepare the δ -lactam, 4,6,6-trimethyl-2-oxo-5,6-dihydro-2*H*-pyridone-3-carbonitrile **3**, the same procedure was used except for cyanoacetic ester which was replaced by cyanoacetamide (0.01 mol) (Fig. 1b).

Synthesis of **2** and **4**

To a solution of **1** (0.01 mol), *N,N*-dimethylformamide dimethyl acetal (0.01 mol) was added. The mixture was stirred for 24 h at room temperature. The residue formed was washed with diethyl ether to provide the enaminolactone, 4-(2-(dimethylamino) vinyl)-6,6-dimethyl-2-oxo-5,6-dihydro-2*H*-pyran-3-carbonitrile **2**. To prepare the enaminolactam, 4-(2-(dimethylamino)vinyl)-6,6-dimethyl-2-oxo-5,6-dihydro-2*H*-pyridone-3-carbonitrile **4**, the same procedure was used.

The synthesized compounds were identified on the basis of data from elemental analysis and ^1H NMR spectroscopy.

Lymphocyte proliferation assay

Peripheral blood was obtained from five healthy non-smoking male (aged 25 years) and five female (aged 25 years) donors, under no medication or food supplements intake and free of any known exposure to genotoxic agents. Fasting venous blood samples were collected in

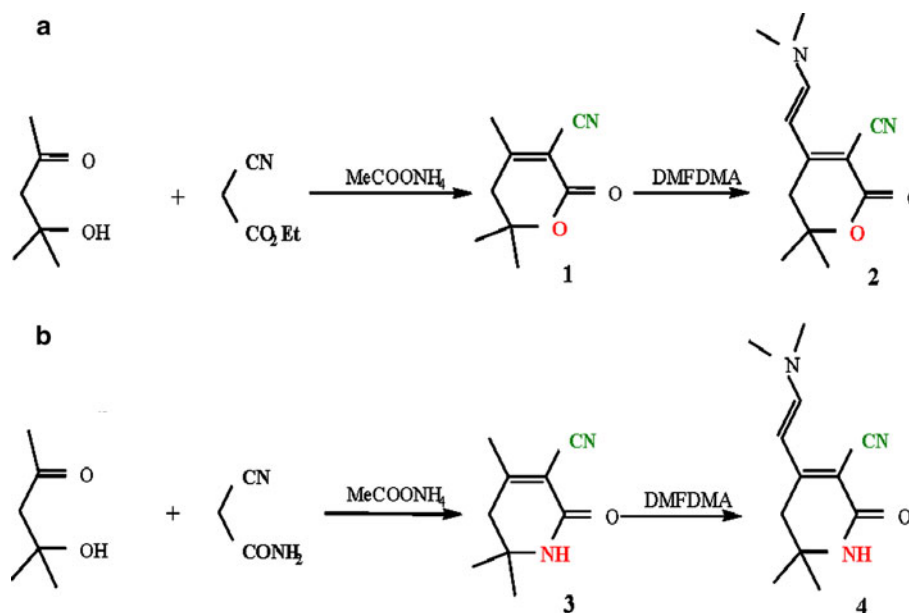


Fig. 1 Chemical synthesis of the compounds used. **a** The reaction of β cetol 4-hydroxy-4-methyl-2-pentanone with cyanoacetic ester and acetate ammonium resulted in the formation of δ-lactone, 4,6,6-trimethyl-2-oxo-5,6-dihydro-2*H*-pyran-3-carbonitrile **1**. The compound **1** reacted with dimethylformamide dimethyl acetal to give 4-(2-(dimethylamino) vinyl)-6,6-dimethyl-2-oxo-5,6-dihydro-2*H*-pyran-3-

carbonitrile **2**. **b** The reaction of β cetol 4-hydroxy-4-methyl-2-pentanone with cyanoacetamide and acetate ammonium resulted in the formation of δ-lactam, 4,6,6-trimethyl-2-oxo-5,6-dihydro-2*H*-pyridone-3-carbonitrile **3**. The compound **3** reacted with dimethylformamide dimethyl acetal to give 4-(2-(dimethylamino) vinyl)-6,6-dimethyl-2-oxo-5,6-dihydro-2*H*-pyridone-3-carbonitrile **4**

heparinized tubes. These samples were used for immediate lymphocyte isolation. The purpose of the study was explained to the volunteer subjects and their consent was obtained. The protocol was approved by the ethical committee of the Tlemcen-University Hospital.

Peripheral blood lymphocytes were isolated from heparinized venous blood using differential centrifugation (400 *g* for 40 min) on a density gradient of Histopaque 1077 (Sigma). The peripheral blood lymphocytes at the interface of plasma and Histopaque were collected and washed twice with RPMI 1640 culture medium (Gibco, USA). After washing and counting, the cells were resuspended in RPMI medium at 4×10^6 cells/ml concentration. For proliferation assay, 4×10^5 cells were cultured in triplicate in 200 μl of medium RPMI 1640 supplemented with 25-mM HEPES buffer, 10% heat-inactivated fetal calf serum, L-glutamine (2 mM), 2-mercaptoethanol (5×10^{-5} M), penicillin (100 UI/ml) and streptomycin (100 μg/ml) with or without mitogen. Concanavalin A (Con A, Sigma, St Louis, MO, USA), a T-cell-specific mitogen was used at 5 μg/ml final concentration. Cultures were grown in 96 flat-bottomed microtiter plates (Nunc, Paris, France) and maintained at 37°C in a 5% CO₂-humidified atmosphere for 48 h. To determine the effects of the compounds synthesized, lymphocytes were incubated with different concentrations of **1**, **2**, **3** and **4**. These

compounds were initially dissolved in DMSO (final solvent concentration <1%) and prepared immediately before use. The concentrations of each compound were adjusted in complete RPMI 1640 culture medium to yield the appropriate final concentration (0.1–10 μM). After incubation, cells were harvested by washing with RPMI 1640 medium. Cell viability was controlled by using a trypan blue exclusion test, and was unaffected by the compound concentrations used in our experiments (greater than 80%). Proliferation was monitored by MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma) assay as described by Mosmann [27]. The absorbance of each sample and control (Con A-free medium) was read on a spectrophotometer at 565 nm.

Stimulation index (SI) was calculated as follows:

$$SI = \left(\frac{\text{Optical density of mitogen-stimulated cells}}{\text{Optical density of non-stimulated cells}} \right) \times 100.$$

Interleukin-2, -4 and INFγ quantification

Aliquots of culture supernatants were used to quantitate interleukins (IL-2, IL-4) and interferon-γ (INFγ) by using commercially available ELISA kits (R & D System, Oxford, UK), as per instructions furnished with. The results

are expressed as pg/ml. The Th1/Th2 ratio was determined as the IFN γ /IL-4 ratio.

Lymphocyte oxidant/antioxidant markers

GSH measurement

Glutathione (GSH) levels were measured using a Bioxytech GSH-400 kit (OXIS International, Inc., Portland, OR, USA). Briefly, cells were resuspended in 500 μ l of 5% (w/v) metaphosphoric acid and were homogenized. After centrifugation of the homogenate at 3000 *g* for 10 min, 100 μ l of the supernatant was transferred to 800 μ l of 200-mM potassium phosphate containing 0.2-mM diethylenetriamine pentacetic acid and 0.025% (w/v) lubrol. Then, 50 μ l of 12-mM chromogenic reagent and 50 μ l of 30% NaOH were added and the mixture was incubated at 25°C for 10 min in the dark. The absorbance at 400 nm was measured, and the GSH concentration was then determined with the GSH standard curve obtained at 400 nm.

Determination of lymphocyte hydroperoxides

To determine markers of lipid peroxidation, hydroperoxides were measured, in sonicated lymphocyte supernatant, by the ferrous ion oxidation-xylene orange assay (Fox2) in conjunction with a specific ROOH reductant, triphenylphosphine (TPP), using a PeroxiDetect kit (Sigma). Calibration was done with standard peroxides such as hydrogen peroxide, measured spectrophotometrically at 560 nm.

Determination of lymphocyte carbonyl proteins

Carbonyl proteins (markers of protein oxidation) were assayed in sonicated lymphocyte supernatant by the 2,4-dinitrophenyl hydrazine reaction as described previously [28].

Micronucleus (MN) assay

The MN assay is used as a fast and reliable assay for detecting genotoxic effects of the compound investigated. For the MN assay, after 24 h of incubation, cytochalasin B (Sigma) was added to the cultures at a concentration of 6 μ g/ml to block cytokinesis. Following additional 24 h of incubation at 37°C, the cells were collected by centrifugation and treated for 3 min with a mild hypotonic solution (75-mM KCl) followed by fixation with a fresh methanol/acetic acid mixture (3:1 v/v). Cells were stained with Giemsa (pH 6.8). Micronuclei were scored in 200 binucleated lymphocytes with well-preserved cytoplasm per incubation, following the established criteria for MN evaluation [29].

Statistical analysis

Data are expressed as mean \pm SD. Statistical analysis was carried out using STATISTICA, version 4.1 (Statsoft, Paris, France). Multiple comparisons were performed using ANOVA followed by the least significant difference (LSD) test. $P < 0.05$ was considered to represent significant statistical differences.

Results

Effects of δ -lactone **1** and δ -lactam **3** and their enaminone derivatives **2** and **4** on in vitro human lymphocyte proliferation

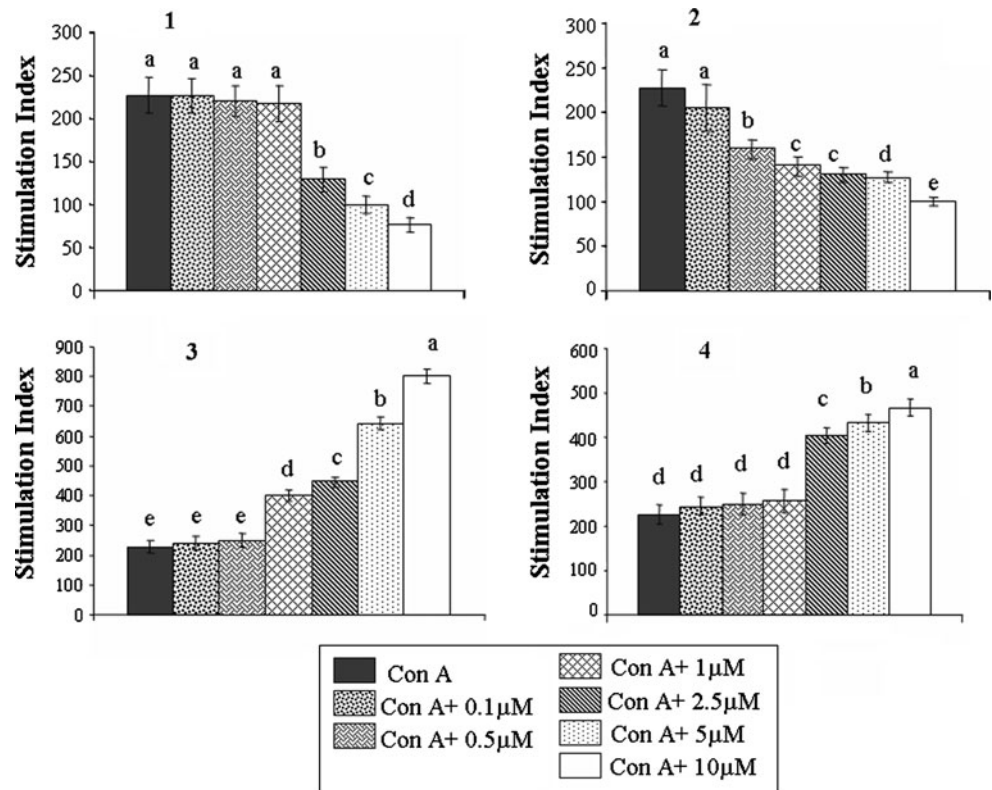
The mean mitogen-stimulated lymphocyte proliferations as expressed by stimulation index co-cultured with or without compounds **1**, **2**, **3** and **4** are shown in Fig. 2. We observed that the compound **1** had no effect on stimulated human lymphocyte proliferation at the concentration of 0.1–1 μ M. However, concentrations of 2.5, 5 and 10 μ M of **1** induced a significant inhibition of Con A-stimulated human lymphocyte proliferation in a dose-dependent manner. The compound **2** induced a significant reduction of stimulation index which occurred at 0.5 μ M and over, reaching a significant maximal inhibition at 10 μ M. Human lymphocytes were more sensitive to **2** at low concentrations but less sensitive at high concentrations compared with their reactivity with **1**.

The effects of the compounds **3** and **4** on lymphocyte proliferation were different from those of **1** and **2**. The compound **3** at concentrations between 0.1 and 10 μ M resulted in an activation of Con A-stimulated lymphocyte proliferation as shown by the increase in the stimulation index in a dose-dependent manner. The compound **4** also induced a significant and progressive activation of Con A-stimulated lymphocyte proliferation. Human lymphocytes were less sensitive to **4** at high concentrations compared with the effect of **3**; the highest SI values were obtained with **3**.

Effects of δ -lactone **1** and δ -lactam **3** and their enaminone derivatives **2** and **4** on in vitro cytokine production

To determine the Th1 and Th2 phenotype, the secretion of cytokines (IL-2, IL-4 and IFN γ) was examined at 48 h of culture (Table 1). The changes in lymphocyte Th-1 (IL-2 and IFN γ) and Th-2 (IL-4) cytokine secretions observed in the presence of compounds **1**, **2**, **3** and **4** were parallel to those seen on the proliferative responses. IL-2, IFN γ and IL-4 productions were significantly decreased by **1** and **2** in

Fig. 2 In vitro influence of different concentrations of δ -lactone **1** and δ -lactam **3** and their enaminone derivatives **2** and **4** on the proliferative response (stimulation index) of human lymphocytes stimulated by mitogen con A. The values are means \pm SD of triplicate assays from ten healthy subjects. Multiple comparisons were performed using ANOVA followed by the least significant difference (LSD) test. Letters *a*, *b*, *c* . . . indicate significant differences obtained with different incubations ($P < 0.05$)



a progressive dose-related manner. In addition, the Th1/Th2 ratio measured as the ratio $\text{IFN}\gamma/\text{IL-4}$ was unaffected by **1** and **2** at concentrations between 0.1 to 2.5 μM . However, at 5 and 10 μM , **1** and **2** induced a significant increase in Th1/Th2 ratio. Human lymphocyte IL-2, $\text{IFN}\gamma$ and IL-4 secretions were significantly enhanced by **3** and **4** in a dose-dependent manner. The $\text{IFN}\gamma/\text{IL-4}$ ratio was unaffected by **3** and **4** at concentrations between 0.1 and 1 μM but it was significantly reduced by these compounds at 2.5–10 μM . Cytokine secretion was more affected by the presence of **1** and **3** in the cultures compared with **2** and **4**.

Effects of δ -lactone **1** and δ -lactam **3** and their enaminone derivatives **2** and **4** on lymphocyte GSH, hydroperoxide and carbonyl protein contents

As shown in Fig. 3, human lymphocyte intracellular glutathione (GSH) levels were not sensitive to **1** and **2** adding in the medium at the concentrations of 0.1–1 μM . However, at higher concentrations (2.5–10 μM), **1** and **2** induced a significant reduction in lymphocyte GSH contents in a dose-dependent manner; the lowest values were obtained with **1**. Lymphocyte intracellular GSH levels were unaffected by **3** and **4** at any concentrations (Fig. 3).

Addition of **1** and **2** in the culture medium at 0.1–1 μM did not affect lymphocyte hydroperoxide (markers of lipid peroxidation) and carbonyl protein (markers of protein

oxidation) levels (Figs. 4 and 5). However, at 2.5–10 μM , **1** and **2** produced significant increases in intracellular hydroperoxide and carbonyl protein levels in a dose-dependent fashion; the highest values were obtained with **1**.

The compounds **3** and **4** have no effects on lymphocyte intracellular hydroperoxide and carbonyl protein levels at low concentrations, whereas they induced a significant increase in hydroperoxide and carbonyl protein contents at high concentrations; the highest values were obtained with **3** (Figs. 4 and 5).

Micronucleus formation in the presence of δ -lactone **1** and δ -lactam **3** and their enaminone derivatives **2** and **4**

The compounds **1** and **2** induced a significant increase in the micronucleus (MN) frequency in human lymphocytes proliferation in a dose-dependent manner (Table 2). The highest values were obtained in the presence of **1** compared with **2**. In contrast, no differences in the MN frequency were found between 0.1 and 1 μM of **3** or between 0.1 and 2.5 μM of **4** and control. Over these concentrations, the presence of **3** and **4** in the cultures was accompanied by a significant increase in the MN frequency; the values were, however, lowest compared with those obtained with **1** and **2**.

Table 1 Th1 (IL-2, INF γ) and Th2 (IL-4) cytokine secretion by Con A-stimulated T lymphocytes in the presence of δ -lactone **1** and δ -lactam **3** and their enaminone derivatives **2** and **4**

	1	2	3	4
IL-2 (Pg/ml)				
Con A	4130 \pm 106 ^a	4130 \pm 106 ^a	4130 \pm 106 ^e	4130 \pm 106 ^d
Con A + 0.1 μ M	4092 \pm 110 ^a	4032 \pm 105 ^a	4282 \pm 98 ^e	4153 \pm 111 ^d
Con A + 0.5 μ M	4081 \pm 105 ^a	2895 \pm 93 ^b	4311 \pm 104 ^e	4204 \pm 116 ^d
Con A + 1 μ M	4056 \pm 117 ^a	2642 \pm 108 ^c	5821 \pm 74 ^d	4233 \pm 108 ^d
Con A + 2.5 μ M	2456 \pm 100 ^b	2433 \pm 71 ^d	6015 \pm 104 ^c	5850 \pm 102 ^c
Con A + 5 μ M	2035 \pm 69 ^c	2416 \pm 82 ^d	6710 \pm 89 ^b	6006 \pm 114 ^b
Con A + 10 μ M	1233 \pm 55 ^d	2052 \pm 67 ^e	7583 \pm 90 ^a	6670 \pm 109 ^a
INF γ (Pg/ml)				
Con A	540.42 \pm 30 ^a	540.42 \pm 30 ^a	540.42 \pm 30 ^e	540.42 \pm 30 ^d
Con A + 0.1 μ M	550.25 \pm 21.22 ^a	506 \pm 22 ^a	562.50 \pm 34 ^e	558.30 \pm 31 ^d
Con A + 0.5 μ M	536.50 \pm 27.26 ^a	388.22 \pm 24.33 ^b	568.39 \pm 27 ^e	562.50 \pm 33 ^d
Con A + 1 μ M	527.74 \pm 29 ^a	350.50 \pm 10.14 ^c	762.53 \pm 30.50 ^d	571.55 \pm 32 ^d
Con A + 2.5 μ M	320.50 \pm 21.66 ^b	327.52 \pm 17.82 ^d	816.24 \pm 23.15 ^c	698.50 \pm 23 ^c
Con A + 5 μ M	257.28 \pm 20.66 ^c	310.66 \pm 12.02 ^d	857.55 \pm 22.40 ^b	760.45 \pm 22 ^b
Con A + 10 μ M	180.56 \pm 22 ^d	285.25 \pm 12.50 ^e	943.32 \pm 26 ^a	800 \pm 20 ^a
IL-4 (Pg/ml)				
Con A	58 \pm 4.40 ^a	58 \pm 4.40 ^a	58 \pm 4.40 ^e	58 \pm 4.40 ^d
Con A + 0.1 μ M	56.78 \pm 3.50 ^a	54 \pm 4.50 ^a	56.54 \pm 4.50 ^e	59.23 \pm 4.02 ^d
Con A + 0.5 μ M	54.15 \pm 4.55 ^a	40.50 \pm 1.50 ^b	59 \pm 4 ^e	60.48 \pm 3.66 ^d
Con A + 1 μ M	50.75 \pm 5.18 ^a	36.43 \pm 2.05 ^c	85 \pm 4.50 ^d	64.08 \pm 5.52 ^d
Con A + 2.5 μ M	30.42 \pm 2.15 ^b	30.05 \pm 2 ^d	104 \pm 3 ^c	88.19 \pm 1.28 ^c
Con A + 5 μ M	22.38 \pm 2.56 ^c	26.40 \pm 1.33 ^e	142 \pm 3.58 ^b	96.77 \pm 1.22 ^b
Con A + 10 μ M	15.50 \pm 2.21 ^d	23.15 \pm 1.11 ^f	175 \pm 3.16 ^a	120.03 \pm 2.35 ^a
INF γ /IL-4				
Con A	9.31 \pm 0.67 ^b	9.31 \pm 0.67 ^b	9.31 \pm 0.67 ^a	9.31 \pm 0.67 ^a
Con A + 0.1 μ M	9.69 \pm 0.73 ^b	9.37 \pm 0.44 ^b	9.94 \pm 0.71 ^a	9.42 \pm 0.55 ^a
Con A + 0.5 μ M	9.90 \pm 0.82 ^b	9.58 \pm 0.62 ^b	9.63 \pm 0.48 ^a	9.30 \pm 0.41 ^a
Con A + 1 μ M	10.39 \pm 0.66 ^b	9.62 \pm 0.71 ^b	8.97 \pm 0.55 ^a	8.91 \pm 0.46 ^a
Con A + 2.5 μ M	10.53 \pm 0.81 ^b	10.89 \pm 0.77 ^b	7.84 \pm 0.50 ^b	7.92 \pm 0.40 ^b
Con A + 5 μ M	11.49 \pm 0.54 ^a	11.76 \pm 0.63 ^a	6.03 \pm 0.47 ^c	7.85 \pm 0.38 ^b
Con A + 10 μ M	11.64 \pm 0.62 ^a	12.32 \pm 0.55 ^a	5.39 \pm 0.43 ^d	6.66 \pm 0.36 ^c

The values are means \pm SD of triplicate assays from ten healthy subjects. Multiple comparisons were performed using ANOVA followed by the least significant difference (LSD) test. Letters a, b, c . . . indicate significant differences obtained with different incubations ($P < 0.05$)

Discussion

In this study, we demonstrate that δ -lactone **1** and δ -lactam **3** and their enaminone derivatives **2** and **4**, synthesized in our laboratory, modulate in vitro lymphocyte proliferation, cytokine secretion and intracellular redox status at the concentrations used in our experiment. To the best of our knowledge, the immunomodulating activity of these compounds has not been documented previously. The immunological properties of other δ -lactones and δ -lactams have been described in the literature and could then be used to improve several diseases associated with malfunctioning of the immune system. Immunosuppression is a common side effect in exposure to various lactones and lactams [11–14, 30]. We also observed an immunosuppressive effect induced by **1** and **2** on lymphocyte response, which

confirms previous reports. However, much to our surprise, **3** and **4** were immunostimulant with anti-inflammatory effect.

The lymphocyte transformation assay is an important tool to measure in vitro mitogen-induced lymphocyte proliferation. This assay offers the opportunity to evaluate an impaired cellular immune response. Pro- and anti-inflammatory cytokines are mediators of the immune system and play an important role in inflammation, acute phase response and disease progression of pathological processes [31]. The lymphocyte transformation assay is based on mitogen stimulation of lymphocytes, and is accepted as a technique to evaluate lymphocyte function. Con A represents the most powerful mitogen for lymphocytes. In our study, the lymphocyte proliferation responses to Con A were affected by the four compounds used, the

Fig. 3 Cellular GSH contents of stimulated T lymphocytes in the presence of δ -lactone **1** and δ -lactam **3** and their enaminone derivatives **2** and **4**. The values are means \pm SD of triplicate assays from ten healthy subjects. Multiple comparisons were performed using ANOVA followed by the least significant difference (LSD) test. Letters *a*, *b*, *c* . . . indicate significant differences obtained with different incubations ($P < 0.05$)

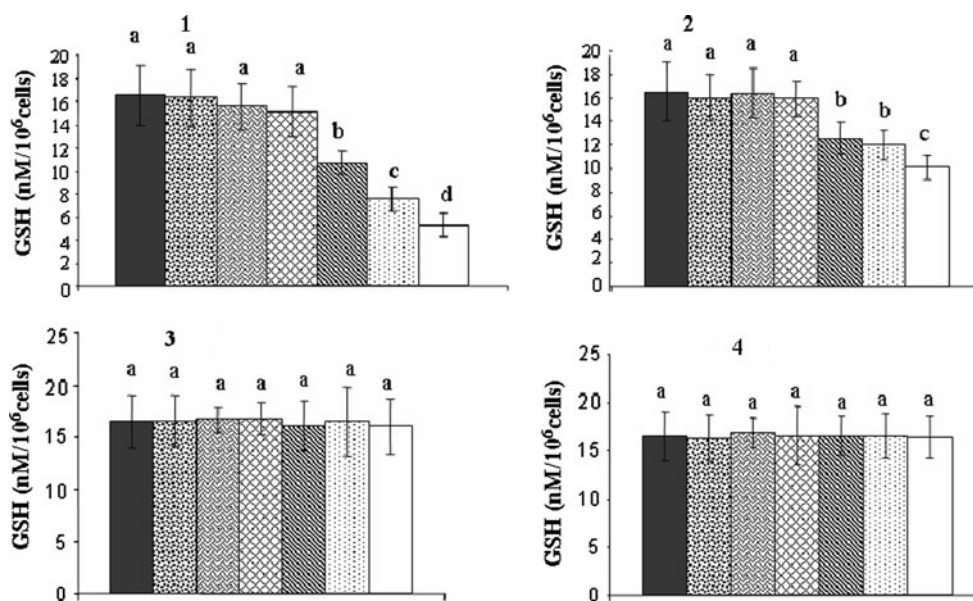
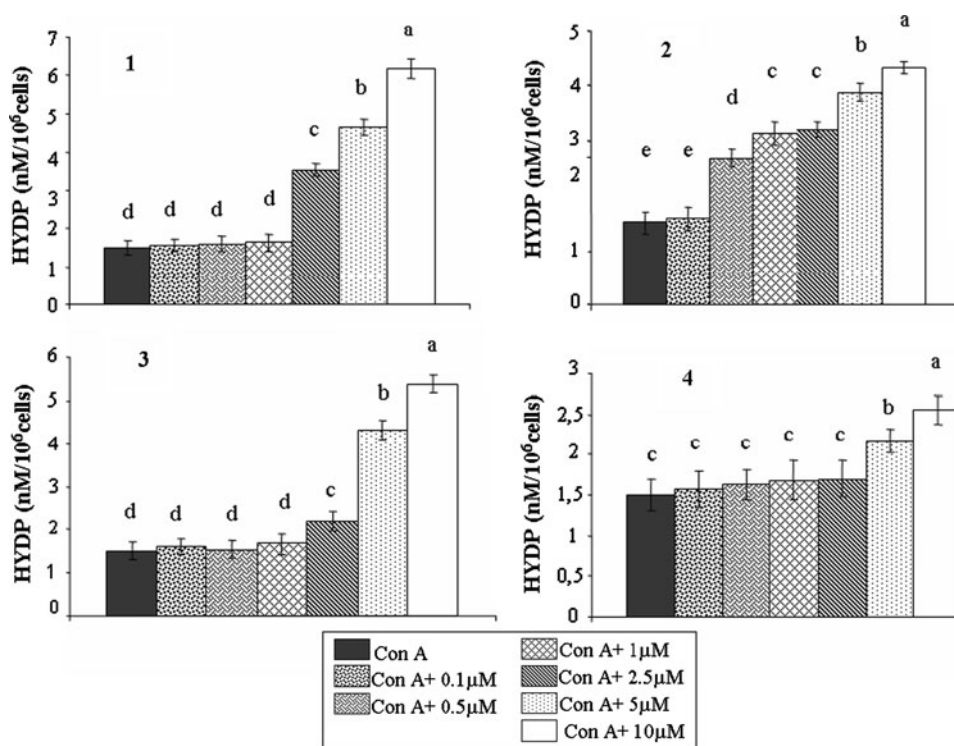


Fig. 4 Cellular hydroperoxide (HYDP) contents of stimulated T lymphocytes in the presence of δ -lactone **1** and δ -lactam **3** and their enaminone derivatives **2** and **4**. The values are means \pm SD of triplicate assays from ten healthy subjects. Multiple comparisons were performed using ANOVA followed by the least significant difference (LSD) test. Letters *a*, *b*, *c* . . . indicate significant differences obtained with different incubations ($P < 0.05$)



effect being related to the presence of δ -lactone or δ -lactam ring. The compounds **1** and **2** decreased mitogen-stimulated lymphocyte proliferation, whereas **3** and **4** increased it, suggesting that **1** and **2** (with δ -lactone ring) are potential immunosuppressive molecules, whereas **3** and **4** (with δ -lactam ring) appear as immunostimulants. We have carried out the condensation of **1** and **3** with dimethylformamide dimethyl acetal to give enamines **2** and **4**. We showed that the introduction of an enamine group into the compound molecule increased its immunomodulatory

activity at low concentrations but attenuated its activity at high concentrations. In fact, the immunosuppression by **2** was more apparent at low concentrations but less aggressive on T-cells at high concentrations compared with **1**. Similarly, the activation of lymphocyte proliferation by high concentrations of **4** was less pronounced than with **3**.

Given the key role of T-helper (Th)1-type and Th2-type cytokines in mounting appropriate immune responses to pathogens and also in human disease [16], it seems important to understand the influence of these compounds

Fig. 5 Cellular carbonyl protein (PCAR) contents of stimulated T lymphocytes in the presence of δ -lactone **1** and δ -lactam **3** and their enaminone derivatives **2** and **4**. The values are means \pm SD of triplicate assays from ten healthy subjects. Multiple comparisons were performed using ANOVA followed by the least significant difference (LSD) test. Letters *a*, *b*, *c* . . . indicate significant differences obtained with different incubations ($P < 0.05$)

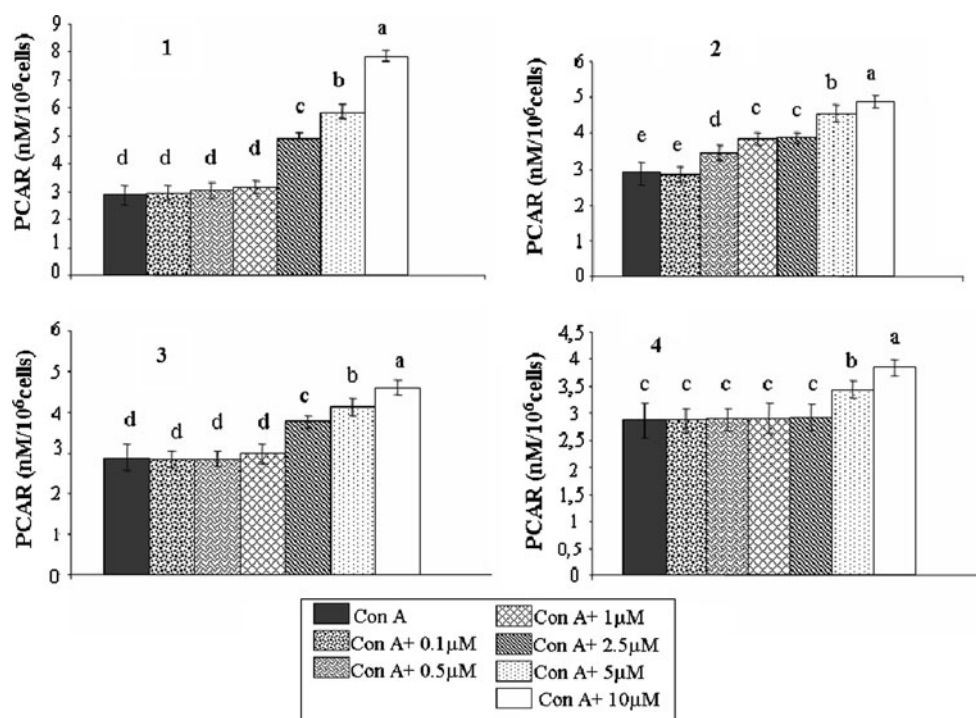


Table 2 Induction of micronuclei by δ -lactone **1** and δ -lactam **3** and their enaminone derivatives **2** and **4** in stimulated T lymphocytes

MN (%)	1	2	3	4
Con A	3 \pm 1.22 ^e	3 \pm 1.22 ^e	3 \pm 1.22 ^d	3 \pm 1.22 ^c
Con A + 0.1 μ M	4.56 \pm 1.32 ^e	4 \pm 1 ^e	3.67 \pm 1.50 ^d	3.30 \pm 1.00 ^c
Con A + 0.5 μ M	7.26 \pm 1.05 ^d	6.33 \pm 0.95 ^d	3.88 \pm 1.45 ^d	3.66 \pm 1.25 ^c
Con A + 1 μ M	8.22 \pm 1.11 ^d	6.74 \pm 1.02 ^d	4.05 \pm 1.01 ^d	4.30 \pm 1.80 ^c
Con A + 2.5 μ M	10.48 \pm 1.02 ^c	8.26 \pm 1.11 ^c	6.25 \pm 1.17 ^c	4.35 \pm 1.50 ^c
Con A + 5 μ M	18.50 \pm 2.31 ^b	10.66 \pm 1.13 ^b	7.88 \pm 1.28 ^b	6.30 \pm 1.30 ^b
Con A + 10 μ M	26.24 \pm 1.52 ^a	15 \pm 1.24 ^a	11.50 \pm 1.35 ^a	8.25 \pm 1.56 ^a

The values are means \pm SEM of triplicate assays from ten healthy subjects. Micronuclei (MN) frequency was calculated for 200 binucleated cells scored for each donor and for each case. Multiple comparisons were performed using ANOVA followed by the least significant difference (LSD) test. Letters *a*, *b*, *c* . . . indicate significant differences obtained with different incubations ($P < 0.05$)

in modulating the production of both Th1-type and Th2-type cytokines. Therefore, in this study, we investigated the production of Th1- and Th2-type cytokines by human lymphocytes cultured in the presence of **1**, **2**, **3** and **4**.

The effects of our compounds on the production of cytokines suggest that they are able to alter the Th1-/Th2-type cytokine balance especially at high concentrations. The compounds **1** and **2** decreased the secretion of IL-2, IL-4 and IFN- γ in activated T-cells and appeared to be particularly potent at skewing this balance away from Th2 toward Th1 at high concentrations (5–10 μ M) suggesting a pro-inflammatory effect. In contrast, **3** and **4** increased the secretion of IL-2, IL-4 and IFN- γ in activated T-cells and altered the balance in production of cytokines away from Th1 and toward Th2 at 2.5–10 μ M suggesting an anti-inflammatory effect. Thus, **1** and **2** provided to

lymphocytes in vitro resulted in decreased cytokine production by lymphocytes, with the strongest effects being observed on Th2 cytokines. The compounds **3** and **4** increased cytokine production by lymphocytes, with the strongest effects being also observed on Th2 cytokines. Although we could not exclude the possibility that other mechanisms such as interference with signal transduction downstream of Con A might also act cooperatively to inhibit or to stimulate T-cell proliferation, we believe that one of the factors contributing to the inhibition or the activation of cell cycle progression is a reduction or a stimulation of IL-2, IL-4, and IFN- γ production in T-cells exposed to the compounds used in this study; Th2 cytokine (IL-4) being the most sensitive cytokine. There have been conflicting reports regarding the modulation of Th1/Th2 balance by other lactones and lactams. Previous reports

suggested that lactones have a more pronounced inhibitory effect on Th1-type responses than on Th2-type responses, favoring of Th2-type responses [32]. Other studies showed that lactones inhibited the differentiation of both Th1 and Th2 cells biased toward Th1 or Th2 [30, 33].

Lactones were shown to be an effective inhibitor of nuclei DNA polymerase activity and of the transcription factor nuclear factor κ B (NF- κ B) [9]. The decreased production of IL-2 in turn leads to decreased numbers of IL-2 receptor and T-cell proliferation [34]. On the other hand, lactones were able to induce cell apoptosis [4], which could explain the reduced T proliferation with **1** and **2**. The precise mechanism of T proliferation enhancement by **3** and **4** is not clear at present, but elevated IL-2 secretion, increased intracellular calcium levels or PKC activation could be affected leading to proliferation, as documented for other lactams [35, 36].

Our results on redox biomarkers in T lymphocytes showed that these cells are submitted to an oxidative stress when exposed to the compounds **1**, **2**, **3** and **4**. GSH has been suggested to regulate lymphocyte proliferation [37]. Our study demonstrated that the compounds **1** and **2** decreased GSH levels and inhibited T-cell proliferation. The compounds **3** and **4** stimulated T-cell proliferation without affecting GSH levels. In addition to decreased IL-2 secretion, depletion in intracellular GSH in the presence of **1** and **2** might also be a factor responsible for the reduction of lymphocyte proliferation. These results are consistent with the work of Hadzic et al. [22], who demonstrated that redox regulation of IL-2 secretion is an obligatory step in T-cell proliferative responses. Indeed, the oxidation of intracellular glutathione is linked to the development of apoptosis [38]. The maintenance of GSH levels in the presence of **3** and **4** might be linked to T-cell activation.

Our results revealed that there was a significant increase in the oxidative stress reflected by an increase in hydroperoxides (lipid peroxidation marker) and carbonyl proteins (protein oxidation marker) in Con A-stimulated human lymphocytes exposed to **1**, **2**, **3** and **4**. The oxidative stress was more pronounced with molecules containing δ -lactone ring (**1** and **2**) compared with those having δ -lactam ring (**3** and **4**). Indeed, the reduction in intracellular levels of GSH in lymphocytes exposed to **1** and **2** was concomitant with the presence of oxidative stress. We showed that the introduction of an enamine group into the compound molecule (**2** and **4**) attenuated the induction of oxidative stress at high concentrations compared with **1** and **3**.

An important potential link between ROS generation and apoptosis has been suggested from evidence in T-cells [18]. The response of the cell to oxidative stress can be very different, depending on the intensity of the stress and its duration, and goes from the stimulation of cell

proliferation to cell death by apoptosis [38]. On the other hand, oxidative stress influences the profile of cytokine secretion in both Th1 and Th2. Low oxidative stress results in lowered Th1 activity and higher Th2 activity [39], in agreement with our findings on compounds **3** and **4**.

Previous results suggest a close relationship between the intracellular uptake and activity of δ -lactones, and an increase in molecular hydrophobicity may be disadvantageous for intracellular uptake (**3**). The higher hydrophobicity of **2** and **4**, compared with **1** and **3**, could explain their attenuated immunological effects at high concentrations, because of decreased intracellular uptake.

Several reports suggested DNA damage in cells exposed to ROS [40–42]. The induction of MN formation (a marker of cytogenetic damage) by exposition to different molecules has been reported by different authors in different test systems [41]. In our study, **1** and **2** induced a significant increase in the MN frequency in human lymphocytes in a dose-dependant manner suggesting a cytotoxic effect which was less pronounced with **2**. The compounds **3** and **4** induced a significant increase in MN frequency only at concentrations greater than 2.5 μ M; **4** being less cytotoxic.

In conclusion, the compounds used in this study displayed immunomodulatory properties depending on the presence of δ -lactone or δ -lactam ring. The δ -lactone **1** and its enaminolactone **2** were immunosuppressive, whereas the δ -lactam **3** and its enaminolactam **4** were immunostimulant, and could be used to provide cell-mediated immune responses for novel therapies in T-cell-mediated immune disorders. The introduction of an enamine group to **1** and **3** seemed to attenuate their immunological properties at high concentrations. In addition, **1**, **2**, **3** and **4** compounds modulated in vitro cytokine secretion with a shift away from Th2 response to Th1 phenotype for **1** and **2**, and a shift away from Th1 response to Th2 phenotype for **3** and **4** at high concentrations. The compounds **1** and **2** had pro-inflammatory effects, whereas **3** and **4** had anti-inflammatory effects. These immunomodulatory properties were, however, accompanied by an increase in lymphocyte intracellular oxidative stress, especially with **1** and **2**.

Acknowledgments This work was supported by the French Foreign Office (International Research Extension Grant TASSILI 08MDU723) and by the Algerian Research Investigation Office (CNEPRU, PNR).

Conflict of interest None.

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