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Changes of in vitro Cytokine Production induced by δ-Lactams

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***Abstract*—**The aim of this work was to study the in vitro effects of δ-lactam 1 and its 4-chlorophenyl derivative 2, on the proliferative responses of human lymphocytes and Th1 and Th2 cytokine secretion. The possible protective role of vitamin E on intracellular stress oxidative induced by these compounds was also investigated. Peripheral blood lymphocytes were isolated using differential centrifugation on a density gradient of Histopaque. They were cultured with mitogen concanavalin A, vitamin E (10 µM) and with different concentrations of the compounds 1 and 2 (0.1 to 10 µM). Proliferation (MTT assay), IL-2, INFγ and IL-4 (Elisa kits), intracellular superoxide anion were determined. 1 and 2 were immunostimulant and increased cytokine secretion with a shift away from Th1 response to Th2. These properties were however accompanied by an increase in intracellular oxidative stress. The presence of vitamin E exhibited protective effects by reducing δ-lactam-induced superoxide anion generation in lymphocytes.

***Keywords*—**Cytokines, δ-Lactams, In vitro Lymphocyte Proliferation, Superoxide Anion

I. Introduction

THIS healthy immune system is held in balanced equilibrium, apparently by the contra-suppressive production of cytokines by T-helper1 (Th1) and T-helper2 (Th2) lymphocyte subsets[1]. T cell activation plays a crucial role in the initiation and regulation of the immune response. Activation of T lymphocytes is a complex process characterized by secretion of the interleukin-2 (IL-2) and by expression of the membrane receptor for IL-2 on the T cell [2]. Stimulation of this receptor by the secreted IL-2 leads in turn to T cell proliferation. IFNγ 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 [3].

Allows you to see the footnotes. Th2 cells secrete IL-4 which is involved in the modulation of antibody production and in the suppression of cell - mediated immunity and inflammation. Deficiency of immune cell production or defective immune cell function can lead to a wide spectrum of immunodeficiency diseases, and the majority of immune diseases are linked to a loss of T-cell homeostasis [4].

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Evaluation of specific biochemical, molecular and immunologic events associated with T cell activation should become a routine analysis in the clinical laboratory in order to identify defects that result in impaired cellular immune functions. Quantifying T cell activation may become useful in monitoring the effect of immunomodulating therapies.

γ lactams have been shown to possess a marked immunotropic activity. γ lactams display immunomodulating properties, including regulation of cell differentiation and effector functions of different immune cells and modulation of cytokine production depending on the molecule used [5]-[7]. Insufficient information is currently available about the effects of δ-lactams on lymphocyte function including proliferation, cytokine production and intracellular redox status although antitumor effects have been previously reported [8]-[9]. We have previously shown that they induced a significant increase in lymphocyte proliferation [10]. However, these immunomodulatory properties were accompanied by an increase in lymphocyte intracellular oxidative stress [10].

To explore new γ lactams with potential useful biological properties, we synthesized γ lactam and its 4-chlorophenyl derivative according to a mechanism described previously [11]-[12] and modified in our laboratory. δ-lactam 1 was obtained by the condensation of 4-hydroxy-4-methyl-2-pentanone with cyanoacetamid in the presence of ammonium acetate [11]. The synthesis of 4-chlorophenyl derivative 2 was carried out by reacting 1 with 4-chlorobenzaldehyde as previously reported by Jansone *et al.* [12]. The aim of this study was to investigate the effect of these γ lactams on in vitro lymphocyte proliferation and cytokine production. In addition, to investigate the possible protective role of vitamin E on intracellular stress oxidative induced by these compounds, lymphocytes were treated with γ lactams in the presence of vitamin E and the production of superoxide anion was measured as an indicator of oxidative stress.

II. Material And Methods

A. Chemical Synthesis

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

Synthesis of 1 and 2

A mixture of βcetol 4-hydroxy-4-methyl-2-pentanone (0.01 mol), cyanoacetamid (0.01 mol), and acetate ammonium (0.005 mol) was heated under reflux for 6 h at 80°C. The precipitate was collected, filtered and washed with diethyl ether to provide the δ-lactam, 4,6,6-trimethyl-2-oxo-5,6-dihydro-2H-pyridone-3-carbonitrile 1 (Fig. a).

Condensation of lactam 1 with 4-chlorobenzaldehyde was carried out in methanol in the presence of a catalytic amount of NaOH (molar ratio of δ-lactam – aldehyde – NaOH 1:1:0.05 respectively at a temperature of 70ºC). The residue formed

was washed with ethanol to provide the 4- (4-chlorostyryl) -6,6-dimethyl- 2-oxo-5,6-dihydro- 2H-pyridone-3- carbonitrile 2 (Figure a).

The synthesized compounds were identified on the basis of data from elemental analysis and IR, ^{1}H NMR,^{13}C NMR spectroscopy. 4,6,6-trimethyl-2-oxo-5,6-dihydro-2H-pyridone-3-carbonitrile 1: White solid; mp:186°C; IR ν_{max}: 2972 ; 3183; 2228; 1660 ; 1618 cm^{-1}; ^{1}HRMN (400 MHz,CDCl3) δ ppm = 1.30 (6H, s); 2.25 (3H, s) ; 2.46 (2H, s); 5.82 (1H,s,NH); ^{13}C RMN (100 MHz,CDCl3) δ ppm = 23.59 ; 29.10 ; 44.97; 77.05; 108.10; 113.81; 160.58; 165.91.

4-(4-chlorostyryl)-6,6-dimethyl-2-oxo-5,6-dihydro-2H-pyridone-3-carbonitrile 2: Yellow solid; mp: 236°C ; IR ν_{max}: 3067.30; 2222; 1668; 1614;1410. cm^{-1} ;^{1}HRMN (400 MHz,CDCl3) δ ppm = 1.31 (6H, s); 2.67 (2H, s); 6.03(1H, s, NH); 7.05-7.35 (2H, d, J = 16Hz); 7.32-7.45(4H, m) ; ^{13}C RMN (100 MHz,CDCl3) δ ppm = 29.11; 37.77; 51.45; 106.47; 114.21; 125.09; 129.34; 130.20; 133.20; 136.80; 139.53; 136.80; 157.88; 161.42.

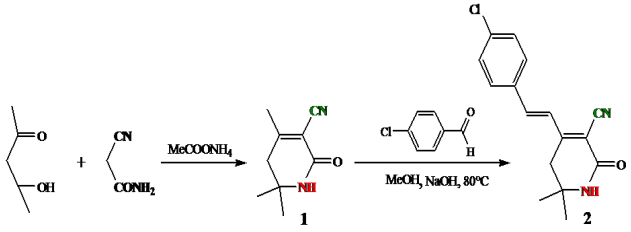

Fig. A Chemical synthesis of the compounds used

B. Lymphocyte Proliferation

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 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 4x10^{6} cells/ml concentration. For proliferation assay, 4x10^{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 (2mM), 2-mercaptoethanol (5x10^{-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% CO_2 humidified atmosphere for 48h. In order to determine the effects of the compounds synthesized, lymphocytes were incubated with different concentrations of 1 and 2.

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 (0.1µM to 10 µM). To determine the effects of vitamin E, 10 µM vitamin E were added in the culture medium. 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 (over 80%). Proliferation was monitored by MTT [3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma) assay as described by Mosmann [13]. The absorbance of each sample and control (ConA- free medium) was read on a spectrophotometer at 565 nm.

Stimulation index (PI) was calculated as follows:

SI = (optical density of mitogen-stimulated cells / optical density of non-stimulated cells) X 100.

C. 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 IFNγ/IL-4 ratio.

D. Lymphocyte Superoxide Anion Determination

The generated superoxide anion ($O_2^{\bullet-}$) was quantified according to the method of Ramanathan et al. [14] based on the reduction of nitroblue tetrazolium (NBT) to monoformazan by $O2^{\bullet-}$. The yellow color of the reduced product was measured spectrophotometrically at 550 nm.

E. Statistical Analysis

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

III. Results

A. Effects of δ-Lactam 1 and its 4-Chlorophenyl Derivative 2 on In vitro Human Lymphocyte Proliferation

The mean mitogen stimulated lymphocyte proliferations as expressed by stimulation index, co-cultured with or without compounds 1 and 2 or in the presence of vitamin E are shown in Fig. 2. We observed that the compound 1 at concentrations between 0.5 µM and 10 µM 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 2 also induced a significant and progressive

activation of Con A-stimulated lymphocyte proliferation. Human lymphocytes were less sensitive to 2 at high concentrations compared to the effect of 1; the highest SI values were obtained with 1. The presence of vitamin E in the medium potentiated the stimulatory effects of the compounds 1 and 2 upon lymphocyte proliferation.

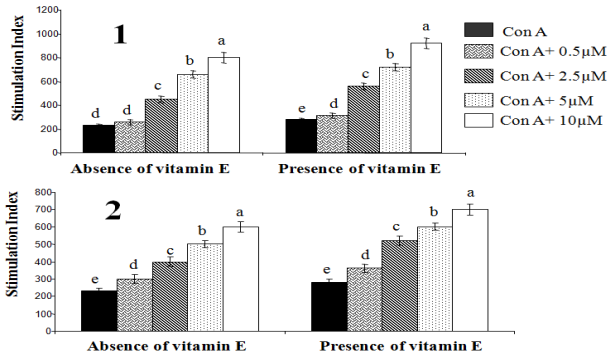

Fig. b In vitro influence of different concentrations of δ-lactam 1 and its 4-chlorophenyl derivatrive 2 on the proliferative response (stimulation index) of human lymphocytes stimulated by mitogen con A in the absence or the presence of vitamin E in the medium. The values are means ± SD of triplicate assays from 10 healthy subjects. Multiple comparisons were performed using ANOVA followed by the least significant difference (LSD) test. a, b, c ... indicate significant differences obtained with different incubations (P< 0.05)

B. Effects of δ-Lactam 1 and Its 4-Chlorophenyl Derivative 2 on In vitro Cytokine Production

To determine the Th1 and Th2 phenotype, the secretion of cytokines (IL-2, IL-4, INFγ) was examined at 48 h of culture (TABLE I). The changes in lymphocyte Th-1 (IL-2 and IFNγ) and Th-2 (IL-4) cytokine secretions observed in the presence of compounds 1 and 2 were parallel to those seen on the proliferative responses. Human lymphocyte IL-2, IFNγ and IL-4 secretions were significantly enhanced by 1 and 2 in a dose dependent manner. To determine whether δ-lactam exposure induced a shift in the Th1/Th2 balance, we calculated the ration between the production of IFNγ, the prototypic Th1 cytokine, and the production of IL-4, the prototypic Th2 cytokine, at different incubations. The IFNγ/IL-4 ratio was unaffected by 1 and 2 at concentration 0.5 µM but it was significantly reduced by these compounds at 2.5 to 10 µM. IL-2 and IFNγ secretion was equally affected by compounds 1 and 2. However, IL-4 secretion was more increased and IFNγ/IL-4 ratio was more decreased by the presence of 2 in the cultures compared to 1. The addition of vitamin E potentiated the effects of 1 and 2 on cytokine production with a further decrease in IFNγ/IL-4 ratio. The reduction of IFNγ/IL-4 ratio by 2 was accentuated in the presence of vitamin E in the culture.

C. Effects of δ-Lactam 1 and Its 4-Chlorophenyl Derivative 2 on Lymphocyte Superoxide Anion Production

1 and 2 produced significant increases in intracellular superoxide anion levels in a dose dependent fashion; the highest values were obtained with 1 (Fig. c). The addition of vitamin E in the culture medium attenuated the effects of 1 and 2 on superoxide anion production. In fact, lymphocytes treated with δ-lactams produced less $O2^{\bullet-}$ in the presence of vitamin E compared to those cultured without vitamin E.

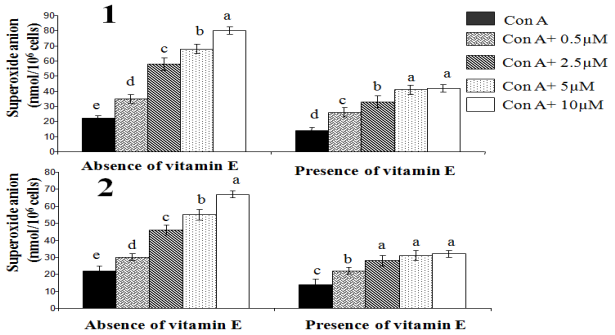

Fig. c Cellular superoxide anion contents of stimulated T lymphocytes in the presence δ-lactam 1 and its 4-chlorophenyl derivatrive 2. The values are means ± SD of triplicate assays from 10 healthy subjects. Multiple comparisons were performed using ANOVA followed by the least significant difference (LSD) test. a, b, c ... indicate significant differences obtained with different incubations (P< 0.05)

TABLE I
TH1 (IL-2, INFΓ) AND TH2 (IL-4) CYTOKINE SECRETION BY CONA – STIMULATED T LYMPHOCYTES IN THE PRESENCE OF Δ-LACTAM 1 AND ITS 4-CHLOROPHENYL DERIVATRIVES 2, WITH OR WITHOUT VITAMIN E IN THE MEDIUM

	1	2
IL-2 (Pg/mL)		
ConA	4130 ± 106 ^{i}	4130 ± 106 ^{j}
ConA+ Vitamin E	4450 ± 77 ^{h}	4450 ± 77 ^{i}
ConA+ 0.5µM	4311 ± 104 ^{h}	5300 ± 106 ^{h}
ConA+ 0.5µM+Vitamin E	5000 ± 102 ^{g}	5845 ± 100 ^{g}
ConA+2.5µM	6015 ± 104 ^{f}	6050 ± 84 ^{f}
ConA+ 2.5µM+Vitamin E	6400 ± 100 ^{e}	6733 ± 80 ^{e}
ConA+5µM	6710 ± 89 ^{d}	7000 ± 86 ^{d}
ConA+ 5µM+Vitamin E	7005 ± 103 ^{c}	7372 ± 88 ^{c}
ConA+10µM	7583 ± 90 ^{b}	7650 ± 102 ^{b}
ConA+ 10µM+Vitamin E	7800± 88 ^{a}	8450 ± 101 ^{a}
INFγ (Pg/mL)		
ConA	540.42 ± 30 ^{c}	540.42 ± 30 ^{e}
ConA+ Vitamin E	560.50 ± 50 ^{c}	560.50 ± 50 ^{e}
ConA+ 0.5µM	568.39 ± 27 ^{c}	635 ± 42 ^{d}
ConA+ 0.5µM+Vitamin E	600.50 ± 30 ^{c}	675 ± 50 ^{d}
ConA+2.5µM	816.24 ± 23.15 ^{b}	789 ± 40 ^{c}
ConA+ 2.5µM+Vitamin E	848 ± 31 ^{b}	855 ± 50 ^{b}
ConA+5µM	857.55 ± 22.40 ^{b}	900 ± 23 ^{b}
ConA+ 5µM+Vitamin E	906.51 ± 28 ^{a}	965 ± 25 ^{a}
ConA+10µM	943.32 ±26 ^{a}	980 ± 20 ^{a}
ConA+ 10µM+Vitamin E	986.24 ± 25 ^{a}	995 ± 35 ^{a}
IL-4 (Pg/mL)		
ConA	58 ± 4.40 ^{i}	58 ± 4.40 ^{g}
ConA+ Vitamin E	66 ± 5 ^{h}	66 ± 5 ^{g}
ConA+ 0.5µM	59 ± 4 ^{i}	75 ± 6 ^{f}
ConA+ 0.5µM+Vitamin E	75 ± 6 ^{g}	85 ± 7 ^{f}
ConA+2.5µM	104 ± 3 ^{f}	122 ± 5 ^{e}
ConA+ 2.5µM+Vitamin E	135 ± 5 ^{e}	156 ± 5 ^{d}

ConA+5µM	142 ± 3.58 [d]	174 ± 6 [c]
ConA+ 5µM+Vitamin E	167 ± 5 [c]	198.55 ± 8 [b]
ConA+10µM	175 ± 3.16 [b]	220 ± 7 [a]
ConA+ 10µM+Vitamin E	216 ± 7 [a]	245 ± 5 [a]
INFγ/IL-4		
ConA	9.31 ± 0.67 [a]	9.31 ± 0.67 [a]
ConA+ Vitamin E	8.49 ± 0.62 [a]	8.49 ± 0.62 [a]
ConA+ 0.5µM	9.63 ± 0.48 [a]	8.46 ± 0.50 [a]
ConA+ 0.5µM+Vitamin E	8.01 ± 0.50 [b]	7.94 ± 0.45 [b]
ConA+2.5µM	7.84 ± 0.50 [c]	6.47 ± 0.38 [c]
ConA+ 2.5µM+Vitamin E	6.28 ± 0.41 [d]	5.48 ± 0.40 [d]
ConA+5µM	6.03 ± 0.43 [d]	5.17 ± 0.35 [d]
ConA+ 5µM+Vitamin E	5.42 ± 0.33 [e]	4.86 ± 0.27 [d]
ConA+10µM	5.39 ± 0.51 [e]	4.45 ± 0.31 [e]
ConA+ 10µM+Vitamin E	4.56 ± 0.46 [f]	4.06 ± 0.28 [e]

The values are means ± SD of triplicate assays from 10 healthy subjects. Multiple comparisons were performed using ANOVA followed by the least significant difference (LSD) test. a, b, c ... indicate significant differences obtained with different incubations (P< 0.05)

IV. Discussion

In the present study, we demonstrate that δ-lactam 1 and its 4-chlorophenyl derivative 2, synthesized in our laboratory, modulate in vitro lymphocyte proliferation, cytokine secretion and intracellular superoxide anion production at the concentrations used in our experiment. We have previously showed the immunostimulant effect of the compounds 1, but the immunomodulating activity of its 4-chlorophenyl derivative 2 has not been documented previously. Our results showed that 1 and 2 were immunostimulant with anti-inflammatory effect. These immunological properties were accompanied by an increase in superoxide anion production, especially with 1. Vitamin E exhibited protective effects in human lymphocytes by inhibiting δ-lactam - induced superoxide anion generation, especially with 2. 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 [15]. 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 compounds used, the effect being related to the presence of δ-lactam ring. 1 and 2 increased mitogen stimulated lymphocyte proliferation, suggesting that 1 and 2 (with δ-lactam ring) appear as immunostimulants, in accordance with our previous findings [10]. We have carried out the condensation of 1 with 4-chlorobenzaldehyde to provide the 4-chlorophenyl derivative 2. We showed that the introduction of the chlorophenyl group into the compound molecule attenuated its activity at high concentrations. In fact, the activation of lymphocyte proliferation by high concentrations of 2 was less pronounced than with 1. One theory of immune regulation involves homeostasis between Th1 and Th2 activity. The role of Th1/Th2 balance in health and disease is well established now. 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 increased 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 Th1 toward Th2 at high concentrations suggesting an anti-inflammatory effect. Thus, 1 and 2 increased cytokine production by lymphocytes, with the strongest effects being observed upon Th2 cytokines, especially in the presence of 2. 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 stimulate T-cell proliferation, we believe that one of the factors contributing to the activation of cell cycle progression is 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 lactams. Previous reports demonstrated antiinflammatory properties in vitro via suppression of the production of the proinflammatory cytokine TNF-α [16]. The precise mechanism of T proliferation enhancement by 1 and 2 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 [17]-[18]. Our results on redox biomarkers in T lymphocytes showed that these cells are submitted to an oxidative stress when exposed to the compounds 1 and 2. Our study demonstrated that the compounds 1 and 2 increased superoxide anion production in human lymphocytes. We have previously reported that the compound 1 induced intracellular oxidative stress via an increase in lymphocyte hydroperoxide and carbonyl protein levels [10]. The introduction of the chlorophenyl group into the compound 1 to provide 2 did not affect the δ-lactam induced superoxide anion generation but it attenuated the intracellular oxidative stress induced by 2 compared to 1. An important potential link between reactive oxygen species generation and apoptosis has been suggested from evidence in T-cells [19]. 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 [20]. 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 [21], in agreement with our findings on compounds 1 and 2. In our study, addition of vitamin E in the culture significantly increased lymphocyte proliferation and cytokine production both in the presence and absence of the compounds 1 and 2. In addition, vitamin E attenuated the δ-lactam induced superoxide anion generation, especially in the presence of 2. Previous studies demonstrated that in vivo vitamin E supplementation

was not only able to promote T-lymphocyte proliferation, but also effectively suppressed the oxidative stress on the lymphocytes [22]. It has been demonstrated that vitamin E increased in vitro lymphocyte proliferation and protected against cytotoxic effects of various chemical molecules, including a decrease in intracellular oxidative stress [23]-[24]. In the presence of vitamin E, the compounds 1 and 2 induced more efficient immunostimulatory and anti-inflammatory effects with reduced intracellular superoxide anion production.

In conclusion, the compounds used in this study displayed immunomodulatory properties depending on the presence of δ-lactam ring. The δ-lactam 1 and its 4-chlorophenyl derivative 2 were immunostimulants. In addition, 1 and 2 compounds modulated in vitro cytokine secretion with a shift away from Th1 response to Th2 phenotype suggesting anti-inflammatory effects. These immunomodulatory properties were however accompanied by an increase in lymphocyte intracellular oxidative stress. Supplementation with vitamin E exhibited protective effects in human lymphocytes by reducing δ-lactam -induced superoxide anion generation. The compounds 1 and 2 could be used to provide cell mediated immune responses for novel therapies in T cell mediated immune disorders in combination with antioxidant supplementation.

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