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Effect of abietane diterpenes from *Plectranthus grandidentatus* on T- and B-lymphocyte proliferation

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Abstract—Five known abietane diterpenes of the royleanone and coleon type, namely, fatty acid esters of 7α-acyloxy-6β-hydroxyroyleanone (**1**), grandidone A (**2**), 7α-acetoxy-6β-hydroxyroyleanone (**3**), 6β,7α-dihydroxyroyleanone (**4**) and coleon U (**5**), isolated from *Plectranthus grandidentatus*, were evaluated for their effect on the proliferation of human lymphocytes induced by the mitogen PHA. All except **4**, showed a dose-dependent suppressor effect, with **3** yielding the most potent antiproliferative activity, followed by **5**. These two compounds, that represent diterpenes of the royleanone and coleon type respectively, were also shown to be potent inhibitors of mouse splenocyte proliferation induced by ConA or LPS mitogens. However, the sensitivity of ConA-stimulated splenocytes to their suppressive effect was higher, suggesting a preferential inhibition of T-lymphocyte proliferation. The antiproliferative activity of **3** seemed to be exerted without affecting the expression of the lymphocyte activation marker CD69. On the contrary, **5** was shown to reduce the expression of CD69 of $TCD8^+$ and B-cells, suggesting a relationship between its antiproliferative effect and the expression of this early marker of activation on these cell populations. The capacity of **5** to induce apoptosis on ConA-stimulated splenocytes could also be related with its antiproliferative activity.

1. Introduction

Several *Plectranthus* species (Lamiaceae) have been used in traditional medicine. The chemistry of *Plectranthus* remains relatively unknown, but the more common secondary metabolites in this genus are diterpenoids, the majority of which are abietanes.[1] In previous work, we reported the isolation of abietane diterpenes, of the royleanone and coleon type, from *P. hereroensis* and *P. grandidentatus* and demonstrated their antibacterial, antifungal and antitumor activity.[2–4] Abietane diterpenes have been frequently isolated from other plant genus and some have cytotoxic, antibacterial, antitumor-promoting and antioxidant activities,[5–8] but to our knowledge they have not been studied for immunomodulatory activity.

As part of our search for new compounds, from synthetic and natural origin, with potential immunomodulatory activity we have recently demonstrated that some synthetic derivatives — catechols — from abietic acid, as well as carnosic acid are potent inhibitors of the proliferation of human lymphocytes induced by phytohaemagglutinin (PHA) mitogen.[9] The aim of the present work was to investigated the effect of five natural known abietane diterpenes, isolated from *P. grandidentatus* Gürke, namely fatty acid esters of 7α-acyloxy-6β-hydroxyroyleanone (**1**), grandidone A (**2**), 7α-acetoxy-6β-hydroxyroyleanone (**3**), 6β,7α-dihydroxyroyleanone (**4**) and coleon U (**5**) on the proliferation of human lymphocytes induced by PHA. The effect of royleanone **3** and coleon **5** was further evaluated on the proliferation of mouse splenocytes in response to the

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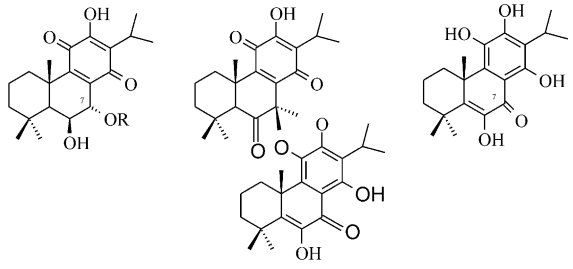
T-cell specific mitogen, ConA and to the B-cell specific mitogen, LPS. Further studies were carried out to determine if the antiproliferative activity exhibited by these two diterpenes was correlated with an alteration of expression of CD69, the earliest marker of lymphocyte activation, or with the apoptosis phenomenon. To the best of our knowledge, this is the first report on the effect of abietanes on the proliferation of B- and T-lymphocytes, CD69 expression and apoptosis.



1 R= fatty acid chain
3 R= Ac
4 R= H

2 **5**

2. Results

2.1. Effect of 1–5 on the PHA-induced proliferation of human lymphocytes

The effect of **1**–**5** on the mitogenic response of peripheral human lymphocytes to PHA was evaluated and the results, given in concentrations that cause 50% inhibition (IC_{50}) of proliferation, are summarised in Table 1. Diterpenes **1**–**3** and **5** inhibited the PHA-induced proliferation of human lymphocytes, in a dose-dependent way (data not shown), while **4** showed no antiproliferative effect even when tested at 50 μM. Royleanone **3** was the most potent diterpene tested exhibiting a strong inhibitory effect ($IC_{50} = 3.8$ μM) that was only 10-fold less potent than cyclosporin, the well-known immunosupressor, used as positive control. This was followed in potency by coleon **5** that showed a moderate antiproliferative effect. Diterpenes **1** and **2** were shown to be poor inhibitors ($IC_{50} > 20$ μM).

2.2. Effect of royleanone 3 and coleon 5 on the proliferation of B- and T-mouse lymphocytes

Given the interesting antiproliferative effects of **3** and **5** on human lymphocytes and since they represented two different types of diterpenes, respectively the royleanone and coleon types, they were further studied in order to evaluate how they affect the proliferation of the two lymphocyte populations, B- and T-cells. BALB/c mouse splenocytes were used, instead of peripheral human lymphocytes, because the percentage of B- and T-lymphocyte is quite similarly in the spleen (60% vs 40%, respectively) compared to the percentage of these two lymphocyte populations in the human peripheral blood (10% vs 90%, respectively). Splenocytes were stimulated either with the T-cell specific mitogen, ConA or with the B-cell specific mitogen, LPS. The effects (IC_{50}) of **3** and **5** on the mitogenic response of splenocytes to these two different mitogens are presented in Table 2. Results showed that both diterpenes strongly inhibited the proliferation of spleen cells induced either by ConA or LPS. However, looking at the IC_{50} values it can be noted that they were more potent inhibitors of the proliferative response of splenocytes induced by ConA than induced by LPS. For **5** the sensitivity of ConA-stimulated splenocytes to its inhibitory effect was shown to be significantly higher than that presented by LPS-stimulated splenocytes ($IC_{50} = 2.96$ μM vs 7.84 μM, respectively; $p < 0.05$).

The possibility of the antiproliferative effect of **3** and **5** being related to a toxic effect was ruled out since non-stimulated and stimulated splenocytes, exposed to the IC_{50} concentrations of these diterpenes, presented viabilities that ranged from 75 to 94%.

From these observations it can be concluded that royleanone **3** and coleon **5** are potent inhibitors of mouse T- and B-lymphocytes proliferation but T-cells are more sensitive to the suppressive effect of these diterpenes.

2.3. Effect of 3 and 5 on the expression of CD69 by T- and B- mouse lymphocytes

Further experiments were carried out to evaluate if the antiproliferative effect of **3** and **5** was related with the expression of CD69, the earliest marker of lymphoide cell activation. For this aim, splenocytes were exposed to the IC_{50} concentrations of **3** and **5** (4.62 and 6.10 μM or 2.96 and 7.84 μM, respectively; see Table 2) and stimulated either with Con A or LPS for 20 h, the time corresponding to the maximum expression of CD69 on mouse splenocytes (data not shown). Non-stimulated spleen cells were also treated with the same concentra-

Table 1. Effect of abietane diterpenes **1**–**5** on the PHA-induced proliferation of human lymphocytes determined by MTT assay

Compd	IC_{50} (μM)
1	31.32 ± 1.63[a]
2	26.63 ± 4.57
3	3.80 ± 1.39
4	> 50
5	12.29 ± 3.23
Cyclosporin A	0.34 ± 0.04

Cyclosporin A was used as positive control. Results are the mean ± SEM of 3–4 independent experiments.
[a] Values in μg/mL.

Table 2. Effect of royleanone **3** and coleon **5** on the proliferation of mouse splenocytes induced by ConA and LPS determined by [^{3}H] thymidine incorporation

Compd	Mitogenic stimuli IC_{50} (μM)	
	ConA	LPS
3	4.62 ± 0.85	6.10 ± 0.06
5	2.96 ± 0.29	7.84 ± 0.41
Cyclosporin A	0.14 ± 0.01	0.58 ± 0.02

Cyclosporin A was used as positive control. Results are the mean ± SEM of one experiment, performed in triplicate, and is representative of three experiments carried out independently.

tions of these diterpenes. The expression of the CD69 antigen was studied in the different lymphocyte populations, T CD4+, T CD8+ and B-cells (Table 3).

As expected, in non-treated control cells, the percentage of CD69 was markedly increased in T CD4+, T CD8+ and B-cells after the Con A or LPS mitogenic stimuli when compared with the percentage of this marker in their correspondent non-stimulated cells. Treatment with **3** was shown not to significantly affect ($p > 0.05$) the expression of the CD69 marker on the surface of both populations either in non-stimulated or stimulated splenocytes. On the contrary, treatment with **5** caused a significant ($p < 0.05$) decrease on the expression of the CD69 marker in T $CD8^+$ and B-cells either in non-stimulated or stimulated splenocytes. No variation of this marker was observed in T $CD4^+$ lymphocytes.

From these results, it can be concluded that royleanone **3** has no effect on the basal levels of the CD69 cell surface marker of non-stimulated splenocytes and does not affect the expression of this molecule on ConA or LPS stimulated splenocytes. However coleon **5** significantly reduces the expression of CD69 on both stimulated and non-stimulated T $CD8^+$ or B-cells, without affecting this expression on T $CD4^+$ cells.

2.4. Effect of 3 and 5 on apoptosis

In order to investigate whether **3** and **5** exerted their antiproliferative activities against ConA stimulated splenocytes by inducing apoptosis, a study on the externalisation of phosphatidylserine (PS), using Annexin V, was performed in treated and non-treated stimulated spleen cells. Different apoptosis detection times were explored (data not shown) with 6 and 24 h being chosen for the apoptosis studies. Results from Figure 1 showed as expected, that in non-treated cells and for both times of incubation, the percentage of apoptosis was higher in stimulated than in non-stimulated splenocytes. Treatment with **3** and **5** for 6 h had no significant effect on the apoptosis of non-stimulated and stimulated splenocytes, when compared with non-treated cells. Although a slight increase was observed in the percentage of apoptotic cells, this was not statistically significant ($p > 0.05$). A 24-h exposure was associated with higher levels of apoptotic cells when compared with control cells, but only a significant variation was observed in stimulated splenocytes treated with **5** ($45.24 \pm 0.77\%$ vs $24.82 \pm 1.96\%$; $p < 0.05$).

3. Discussion

The study of the effect of the five natural abietane diterpenes **1**–**5** on the mitogenic response of human lymphocytes to PHA revealed that all, except **4**, displayed an inhibitory effect on the proliferation of lymphocytes. Comparing the effects of the three related royleanones **1**, **3** and **4** it was concluded that the nature of the substituents at C-7α was decisive for their antiproliferative effect. Royleanone **3** with an acetyl group was found to be the most potent compound while the presence of a hydroxy group in **4** was responsible for the lost of activity. A possible explanation for the strong activity of **3** could be the increase of lipophilicity caused by acetylation. The presence of the fatty acid chain at the same position in royleanone **1**, increasing the lipophilicity even more, was associated with a higher activity. Nonetheless, it exhibited a poor suppressive effect due probably to the hindrance caused by the large fatty chain. The presence of intramolecular hydrogen bondings in coleon U (**5**) or even the possibility of the formation of a quinonoid structure by an oxidative pathway could be possible explanations for the interesting antiproliferative effect of this compound.

Since **3** and **5** exhibited the highest activities and are representatives of two groups of diterpenes (royleanone and coleon respectively), their antiproliferative effects were explored in order to evaluate if they could affect the proliferation of the B- and T-lymphocyte populations equally. For this purpose two mitogenic stimuli, ConA and LPS, responsible for prevalent T- or B-cell responses, were used. They were shown to strongly affect the proliferation of the two lymphocyte populations, but the high sensitivity of the ConA-induced splenocytes to both compounds leads us to conclude that they inhibited T-lymphocytes preferentially. Differences in sensitivity to T- and B-mitogenic stimuli have been described for some flavonoids[10–12] and it was suggested that this difference might reflect utilisation of different pathways of cell activation.[13] Once **3** and **5** inhibited the proliferation induced by either T- or B-

Table 3. Effect of royleanone **3** and coleon **5** on the expression of CD69 of T and B cells from BALB/c mice analysed by flow cytometry

ConA or LPS stimulation	Diterpene treatment	T $CD4^+$ cells (%)	T $CD8^+$ cells (%)	B cells (%)
No	No	1.73 ± 0.26	1.48 ± 0.02	6.77 ± 1.18
No	**3**	1.68 ± 0.36	0.88 ± 0.12[a]	6.46 ± 0.46
No	**5**	1.76 ± 0.34	0.71 ± 0.21[b]	3.74 ± 0.69[b]
Yes	No	16.68 ± 1.03	14.53 ± 1.20	56.42 ± 4.55
Yes	**3**	14.93 ± 1.00	11.91 ± 0.70	44.79 ± 9.82
Yes	**5**	15.95 ± 1.35	9.52 ± 1.63[b]	10.61 ± 0.99[b]

Results show means ± SEM of 2–4 independent experiments, performed with triplicate cultures from a cell pool of three mouse spleens. Viability was superior to 87% throughout the assays as determined by trypan blue assay.

[a] The variation of CD69 expression between royleanone **3** treated non-stimulated T CD8+ cells and control cells was not statistically significant ($p > 0.05$).

[b] A significant difference ($p < 0.05$) in the expression of CD69 was observed between coleon **5** treated non-stimulated or stimulated T CD8+ and B cells and non treated cells.

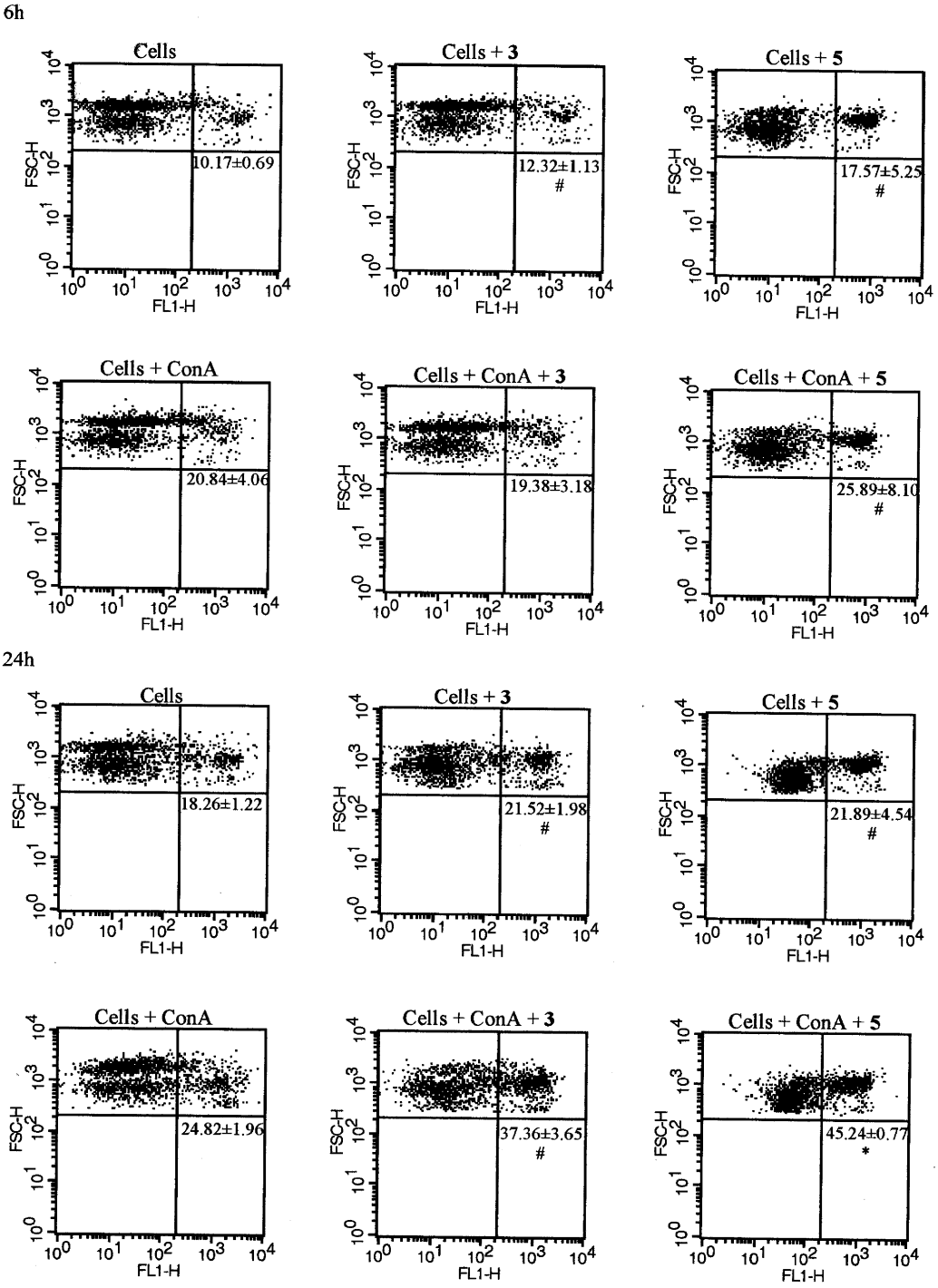


Figure 1. Effect of **3** and **5** on apoptosis in BALB/c splenocytes analysed by flow cytometry. Splenocytes were stimulated or not with ConA and treated or not with **3** or **5**. Viability was always superior to 87% as determined by the trypan blue assay. Analysis of apoptotic cells, performed at 6 and 24 h, refers only to viable cells (PI exclusion). Data is from an experiment, performed with triplicate cultures from a cell pool of three mouse spleens and is representative of five experiments carried out independently. Nonstimulated splenocytes exposed to UV light for 15 min and incubated for 6 or 24 h, used as positive control of cells undergoing apoptosis, showed a percentage of 50 and 64%, respectively. The variation of the percentage of apoptotic cells between treated and non-treated control cells was statistically significant ($^{*}p < 0.05$) or not ($^{\#}p > 0.05$).

stimuli, this could reflect an inhibition of one or more pathways common to T- and B-lymphocyte proliferation. Activation of the transcription factor NF-kB has been shown to be a key component of innate immunity[14] and its inhibition related with the suppression of lymphocyte proliferation.[15] Although the cellular mechanisms responsible for the antiproliferative effect of diterpenes remain to be determined, an interference with NF-kB cannot be excluded since various terpenoids, including diterpenes, have been described as potent inhibitors of NF-kB activation.[14] An interference with the cell signal transduction pathway such as protein kinase C and/or protein tyrosine kinase can also be suggested as one possible mechanism responsible for the

antiproliferative effect of these terpenoids. It is now known that the proliferative signal is generated by members of a family of protein tyrosine kinases (PTKs) that catalyse the phosphorylation of cellular substrates, which in turn lead to T-cell proliferation.[16] Phosphorylation of tyrosine also occurs in B-lymphocyte activation and is an obligatory event in IL-2 secretion.[17] Some terpenoids have shown to inhibit IL-2 production[18,19] and once the production of IL-2 is an essential component in the activation and maintenance of T-cell proliferation, the inhibition of the mitogen-stimulated lymphocyte proliferation by these abietane diterpenes could also be related with the suppression of this interleukin.

Although the role of the early marker of lymphocyte proliferation CD69 in proliferation is not well defined, CD69 seems to be able to transduce signals resulting in gene expression during cell proliferation.[20] In an attempt to find a correlation between its expression and lymphocyte proliferation, the effect of **3** and **5** on the CD69 expression was evaluated on T CD4+, T CD8+ and B-cells. Royleanone **3** did not alter significantly its expression, which indicates that its antiproliferative effect is exerted without interference with this marker. On the contrary, coleon U (**5**) was found to affect CD69 expression on TCD8+ and B-lymphocytes effectively, suggesting that its capacity to inhibit the proliferation of T- and B-cells possibly involves the decrease of this marker.

Finally, it was evaluated if the impairment of lymphocyte proliferation caused by these compounds could induce apoptosis of these cells. No relationship could be established between royleanone **3** and this phenomenon but a significant pro-apoptotic effect was observed in ConA-stimulated cells after a 24 h treatment with coleon **5**. Therefore it is reasonable to assume that **5** exerted its suppressive effect towards T-cells through the involvement of apoptosis.

The different potency presented by **3** and **5** in these two cellular events, CD69 expression and the apoptosis phenomenon, might reflect differences in the mechanism of action underlying the antiproliferative activity of the compounds of royleanone and coleon type.

4. Conclusions

From this work, it can be concluded that these two abietanes (**3** and **5**), of the royleanone and coleon type respectively, strongly suppress the proliferation of T- and B-lymphocytes induced by mitogens, inhibiting preferentially T-cell population. The antiproliferative effect of coleon U (**5**) could be related with its capacity to decrease the expression of CD69 and to induce lymphocyte apoptosis. No significant relationship could be established among these events and the inhibitory effect of royleanone **3**, which might reflect differences in the mechanism of action that underlies the antiproliferative effect of the two groups of compounds. Although this study did not ascertain the cellular mechanism underlying the suppressive effect of the royleanone and coleon type abietane diterpenes, it demonstrated that these compounds can affect the immune functional status and might be considered an attractive candidates for development of immunomodulatory drugs.

5. Experimental

5.1. Isolation of abietanes

Details concerning the extraction, isolation and structure elucidation of the abietane diterpenes **1**–**5** from *P. gradidentatus* have been previously described.[3,4]

5.2. Reagents

Foetal bovine serum (FBS), HEPES and RPMI-1640 medium were obtained from Gibco Invitrogen Corporation (Scotland). [^{3}H]thymidine was purchased from Amersham (Arlington Heights, IL). R-Phycoerythrin (PE)-conjugated hamster anti-mouse CD69, fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 and FITC-conjugated rat anti-mouse CD8 were obtained from BD Biosciences (San Diego, CA). FITC-conjugated goat anti-mouse IgM (anti-μ) and the Annexin V FITC Apoptosis Detection Kit were sourced from PharMingen (San Diego, CA). Unless otherwise indicated all other reagents were obtained from Sigma (St Louis, MO).

5.3. Samples

Stocks solutions of the diterpenes **1**–**5** were prepared in DMSO and stored at −20 °C. The frozen samples were freshly diluted to the desired final concentrations with culture medium prior to the different assays. Final concentrations of DMSO did not interfere with any of the biological activities tested.

5.4. Human peripheral blood mononuclear cells isolation

Human mononuclear cells were isolated from heparinised peripheral blood of healthy volunteers by Histopaque-1077 density centrifugation. Human mononuclear cells were adjusted to $2–3\times10^6$ cells/mL in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine and 50 μg/mL of gentamicin (designated thereafter as culture medium).

5.5. Mouse splenocytes isolation

BALB/c mice were obtained from Harlen Iberica (Spain). Mice were sacrificed by cervical dislocation and spleens were removed and homogenised in a Petri dish. Splenocytes were washed and adjusted to 5×10^6 cells/mL in the culture medium supplemented with 20 mM HEPES.

5.6. MTT-proliferation assay

The effect of diterpenes **1**--**5** on the mitogenic response of human lymphocytes to PHA (10 μg/mL) was evaluated using a modified version of the colorimetric

MTT-assay, previously described by our group.[21] Cyclosporin A was used as a positive control.

5.7. Thymidine proliferation assay

The effect of 7α-acetoxy-6β-hydroxyroyleanone (**3**) and coleon U (**5**) on the proliferation of BALB/c mouse splenocytes induced by the mitogens concanavalin A (ConA, 6 μg/mL) or lypopolyssacharide (LPS, 10 μg/mL) was determined by [^{3}H]thymidine incorporation. Cyclosporin A was used as a positive control. Mouse splenocytes were cultured in 96-well flat-bottom plates and exposed simultaneously to serial concentrations of **3** or **5** and to the different mitogens, ConA or LPS for 48 or 72 h, respectively, at 37 °C. In the last 8 h of incubation 1 μCi of [^{3}H]thymidine was added. At the end of incubation, pulsed cells were harvested on a glass filter 102*256 mm (Skatron, Norway) using a semiautomatic cell harvester (Skatron Instruments, Norway) and allowed to dry. Incorporation of radioactive thymidine was determined by liquid scintillation in a scintillation counter LS 6 500 (Beckman, CA). The concentration of compound giving 50% of inhibition of [^{3}H]thymidine incorporation was calculated comparing the arithmetic mean of counts per min (cpm) of compound exposed cells with that of the non-exposed cells (control cells).

5.8. Trypan blue viability assay

To evaluate a possible toxicity of diterpene **3** and **5** against stimulated and nonstimulated splenocytes, the trypan blue exclusion assay was used. Cells were counted in a hemocytometer and splenocyte toxicity, determined in terms of percentage of viable cells, was excluded when the viability of the compound exposed cells, compared with that of the non-exposed control cells, was greater than 70%.

5.9. Flow cytometry analysis of CD69 expression by splenocytes

Mouse splenocytes, cultured in 96-well flat-bottom plates, were treated with 4.6 μM of compound **3** or 3 μM of compound **5** and stimulated with ConA (6 μg/mL) or treated with 6.1 μM of **3** or 6.8 μM of **5** and stimulated with LPS (10 μg/mL), for 20 h at 37 °C. Nonstimulated splenocytes were also treated with the same concentrations of these compounds. After incubation, plates were placed on ice for 5 min and cells transferred to U-well plates. They were washed and ressuspended in PBS supplemented with 2% FBS (PBS-FBS). Cell suspensions were incubated for 30 min at 4 °C with saturing concentrations of PE-conjugated hamster anti-mouse CD69 (detected by FL2-H) and FITC-conjugated rat anti-mouse CD4 or FITC-conjugated rat anti-mouse CD8 or FITC-conjugated goat anti-mouse Ig M (anti-μ) (detected by FL1-H). After incubation, cells were washed three times with PBS-FBS and centrifuged. Cells were ressuspended in PBS-FBS and propidium iodide (PI, 1 μg) was added just before acquisition. Flow cytometric analysis was performed in a fluorescent activated cell sorter (FACSCalibur, BD Biosciences, San Diego, CA). Lymphocytes were selected on the basis of FSC/SSC values and to viable cells, once propidium iodide was used to exclude the dead cells. To determine the percentage of CD69 in T CD4+, T CD8+ and B cells the different cell populations were positively gated.

5.10. Annexin V apoptosis assays

The detection of phosphatidylserine on the cell surface of mouse splenocytes was performed with the Annexin V FITC Apoptosis Detection Kit. Briefly, non-stimulated and stimulated (ConA; 6 μg/mL) splenocytes, cultured in 96-well flat-bottom plates, were exposed or not to compound **3** (4.6 μM) or **5** (3 μM) for 6 and 24 h. They were then transferred to 96-U-well plates (1×10^6 cell/well), ressuspended in the binding buffer (100 μL) and stained with Annexin V FITC (2 μL) (detected by FL1-H). After 15-min incubation at room temperature in the dark, PI (1 μg) was added and cells were analysed by flow cytometry. Lymphocytes were selected on the basis of FSC/SSC values and to viable cells, once propidium iodide was used to exclude the dead cells. Non-stimulated splenocytes exposed to UV-light for 15 min and incubated as above for 6 and 24 h, were used as positive control of cells undergoing apoptosis.

5.11. Statistics

Results are expressed as mean values ± SEM (standard error of the mean). The statistical significance of the difference between control and treated samples was calculated by the Student's *t*-test. A p value lower than 0.05 was taken to be statistically significant.

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References and notes

1. Abdel-Mogib, M.; Albar, H. A.; Batterjee, S. M. *Molecules* **2002**, *7*, 271.
2. Batista, O.; Duarte, A.; Nascimento, J.; Simões, F.; de la Torre, M. C.; Rodríguez, B. *J. Natl. Prod.* **1994**, *57*, 858.
3. Teixeira, A. P.; Batista, O.; Simões, F.; Nascimento, J.; Duarte, A.; de la Torre, M. C.; Rodríguez, B. *Phytochemistry* **1997**, *44*, 325.
4. Marques, C.; Pedro, M.; Simões, M.; Nascimento, M. S. J.; Pinto, M.; Rodríguez, B. *Planta Med.* **2002**, *68*, 839.
5. Kinouchi, Y.; Ohtsu, H.; Tokuda, H.; Nishino, H.; Matsunaga, S.; Tanaka, R. *J. Natl. Prod.* **2000**, *63*, 817.
6. Ulubelen, A.; Oksuz, S.; Kolak, U.; Bozok-Johansson, C.; Celik, C.; Voelter, W. *Planta Med.* **2000**, *66*, 458.
7. Demetzos, D.; Dimas, K.; Hatziantoniou, S.; Anastasaki, T.; Angelopoulou, D. *Planta Med.* **2001**, *67*, 614.
8. Mei, S.-X.; Jiang, B.; Niu, X.-M.; Li, M.-L.; Yang, H.;

Na, Z.; Lin, Z.-W.; Li, C.-M.; Sun, H.-D. *J. Natl. Prod.* **2002**, *65*, 633.
9. Gigante, B.; Santos, C.; Silva, A.; Curto, M. J. M.; Nascimento, M. S. J.; Pinto, E.; Pedro, M.; Cerqueira, F.; Pinto, M. M.; Duarte, M. P.; Laires, A.; Rueff, J.; Gonçalves, J.; Pegado, M. I.; Valdeira, M. L. *Bioorg. Med. Chem.* **2003**, *11*, 1631.
10. Mookerjee, B. K., Lee, T. P., Logue, G. P., Lippe, H. A., Middleton, E. In *Plant Flavanoids in Biology and Medicine: Biochemical, Pharmacological and Structural-activity Relationships*, Cody V et al.; Alan R. Liss: New York, 1986; p 511.
11. Namgoong, S. Y.; Son, K. H.; Chang, H. W.; Kang, S. S.; Kim, H. P. *Life Sci.* **1993**, *54*, 313.
12. You, K.; Son, K.; Chang, H.; Kang, S.; Kim, H. *Planta Med.* **1998**, *64*, 546.
13. Middleton, E.; Kandaswami, C.; Theoharides, T. *Pharmacol. Rev.* **2000**, *52*, 673.
14. de las Heras, B.; Navarro, A.; Díaz-Guerra, M. J.; Bermejo, P.; Castrillo, A.; Boscá, L.; Villar, A. *Br. J. Pharmacol.* **1999**, *128*, 605.
15. Gao, X.; Xu, Y.; Janakiraman, N.; Chapman, R.; Gautam, S. *Biochem. Pharmacol.* **2001**, *62*, 1299.
16. Rudd, C. E. *Immunol. Today* **1990**, *11*, 400.
17. Stanley, J. B.; Gorczynski, R.; Huang, C.-K.; Love, J.; Mills, J. B. *J. Immunol.* **1990**, *145*, 2189.
18. Duan, H.; Takaishi, Y.; Momota, H.; Ohmoto, Y.; Taki, T.; Tori, M.; Takaoka, S.; Jia, Y.; Li, D. *Tetrahedron* **2001**, *57*, 8413.
19. Takaishi, Y.; Miyagi, K.; Kawazoe, K.; Nakano, K.; Li, K.; Duan, H. *Phytochemistry* **1997**, *45*, 975.
20. Testi, R.; Phillips, J.; Lanier, L. *J. Immunol.* **1989**, *143*, 1123.
21. Gonzalez, M. J.; Nascimento, M. S. J.; Cidade, H.; Pinto, M. M. M.; Kijjoa, A.; Anantachoke, C.; Silva, A.; Herz, W. *Planta Med.* **1999**, *65*, 368.