Anticlastogenic Effect in Human Lymphocytes by the Sodium Salt of 3,4-Secoisopimar-4(18),7,15-trien-3-oic Acid

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Ⓢ Supporting Information

ABSTRACT: The ability of the sodium salt of 3,4-secoisopimar-4(18),7,15-trien-3-oic acid (**1**), a diterpenoid obtained from *Salvia cinnabarina*, to inhibit the genotoxic effect of ethyl methanesulfonate (a clastogenic agent) and colcemid (an aneugenic agent), was studied using a micronucleus assay on cultured human lymphocytes. Cells were treated with **1** before (pretreatment), during (co-treatment), and after (post-treatment) treatment with the mutagens, in order to investigate the type of antimutagenic activity (desmutagenic or bioantimutagenic) manifested. In the range of concentrations tested (0.3–330 μM) **1** reduced significantly the frequency of micronuclei induced by ethyl methanesulfonate, in both pre- and co-treatment protocols (up to 74% and 70% of reduction, respectively), showing an anticlastogenic activity. Conversely, **1** did not inhibit the effect of colcemid in all treatments. The nuclear division index value of lymphocytes was not affected by treatment with **1**, thus demonstrating that the anticlastogenic effect of **1** was not due to a cytotoxic effect. On the basis of the results obtained, it can be hypothesized that **1** exerts its anticlastogenic activity against ethyl methanesulfonate by a desmutagenic mechanism, possibly by chemical inactivation of the mutagen.
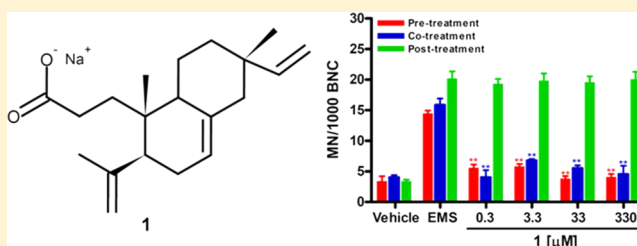
Diterpenes are naturally occurring compounds that exert several biological activities such as antimicrobial, antifeedant, and anti-inflammatory effects.[1−3] Several diterpenes have also been shown to possess pronounced antioxidant, antitumor, and chemopreventive effects.[3−5] For example, the diterpenoids 6α-hydroxysugiol, salvadoriol, and cuzcol, from *Thuja standishii*, *Maytenus cuzcoina*, and *Crossopetalum uragoga*, respectively, were found to be potent antitumor promoter agents in Raji cells.[6,7] Analogously, the abietane diterpene carnosol, a chemical component of *Rosmarinus officinalis* and *Salvia officinalis*, showed promising anticancer and chemopreventive activity in several in vitro models.[8] Finally, two diterpenoids isolated from *Coffea arabica*, cafestol and kahweol, showed a broad range of biochemical effects and are considered responsible for the chemopreventive effects of coffee against several carcinogens.[9,10]

3,4-Secoisopimar-4(18),7,15-trien-3-oic acid is a secoisopimarane diterpenoid isolated from the aerial parts of *Salvia cinnabarina* M. Martens et Galeotti (Lamiaceae)[11] and was characterized chemically by Romussi et al., with an extractive yield, from the total surface exudate of aerial parts, of about 37%.[12] This compound was shown to possess several biological activities, both in vitro and in vivo, such as spasmolytic and anxiolytic effects.[13] In a previous in vitro study, the sodium salt of 3,4-secoisopimar-4(18),7,15-trien-3-oic acid (**1**) was shown to possesses a strong antimutagenic activity, in a bacterial reverse mutation assay (Ames test), against several mutagens, particularly aromatic amines and nitroarenes.[14]
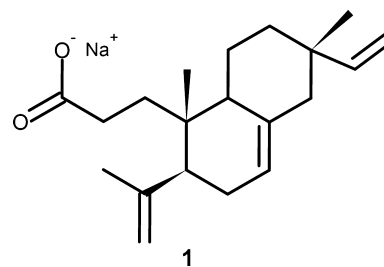
It is well known that the first step in characterizing the genotoxicity/antigenotoxicity of a substance is to study its effect on different genomic end points (e.g., gene mutations, chromosomal damage) by combining both bacterial and mammalian in vitro assays.[15] A very effective tool is represented by the combination of an Ames test, which detects gene mutations, and the in vitro micronucleus test, which detects both a clastogenic and an aneuploidogenic effect.[15]

On the basis of this evidence and in order to study in more detail the properties of **1**, the present study was aimed at

Pre-treatment

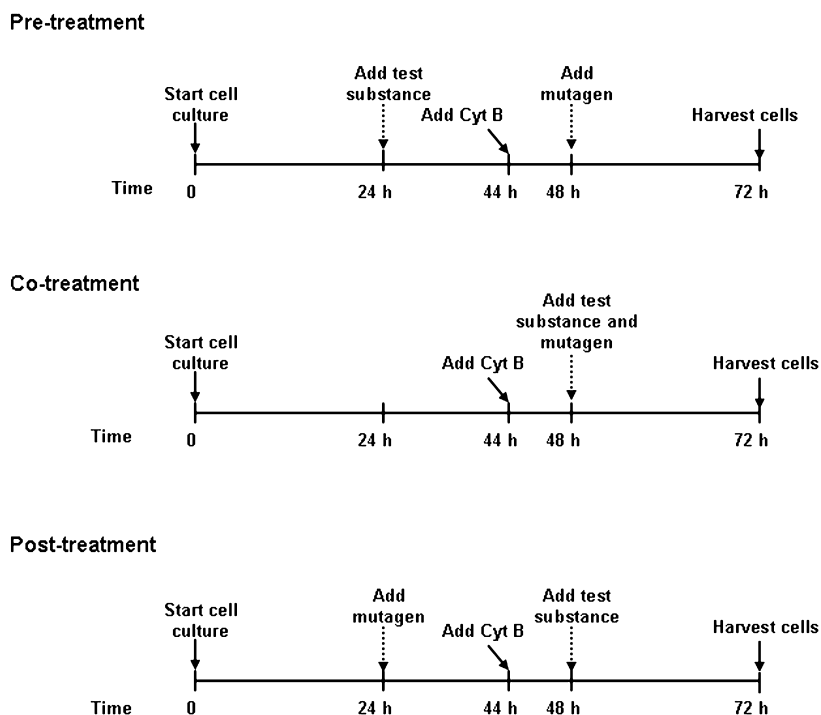## Co-treatment

Post-treatment

Figure 1. Schematic diagram of treatment protocols used to test the antigenotoxic effect of **1**. Cyt B: cytochalasin B.

evaluating the potential protective effects of this test compound at a chromosomal level by the micronucleus assay in cultured human lymphocytes. In particular, the protective activity of **1** was assayed against the genotoxic damage induced by two different mutagens, ethyl methanesulfonate, a clastogenic agent,[16,17] and colcemid, an aneugenic agent.[18,19] In order to investigate the possible mechanism of action, lymphocyte cultures were treated with compound **1** before (pretreatment), during (co-treatment), and after (post-treatment) treatment by the mutagens.

■ RESULTS AND DISCUSSION

Preliminary assays showed that **1**, in the range of concentrations tested (0.3−330 μM) on the cultured human lymphocytes, neither affected the nuclear division index (NDI) nor increased the frequency of micronuclei (MN), with respect to the control cultures. These results excluded both a cytotoxic and a genotoxic effect (Figure 2) of **1** and allowed evaluation of this substance for its possible antimutagenic effect.

In the antimutagenicity assay, **1** reduced in a statistically significant manner the frequency of MN induced by ethyl methanesulfonate, in both the pre- and co-treatment protocols (Table 1). In contrast, it did not modify the effect of colcemid. These results suggest that **1** possesses an anticlastogenic but not an aneugenic activity. The anticlastogenic effect was specific and not due to any cytotoxic effect on lymphocytes, because, as stated, the NDI value obtained for the cultures treated with **1** did not statistically differ from those of the control culture, in all protocols used (Table 1).

The anticlastogenic effect of **1** was not concentration-dependent, and the highest decrease in the ethyl methanesulfonate-induced frequency of micronuclei occurred at 33 μM in the pretreatment (74%) and at 0.3 μM in the co-treatment (70%) modes (Table 1). The minimum decrease occurred at 3.3 μM, using both pretreatment (59%) and co-treatment
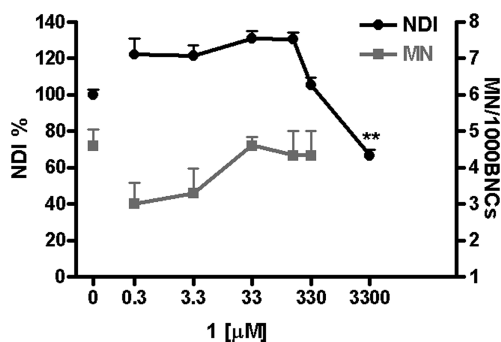

Figure 2. Effects of **1** on micronuclei frequency and on nuclear division index in cultured peripheral lymphocytes. Values are expressed as mean ± SEM (n = 6). NDI % was obtained from the ratio percent of the control untreated. MN: micronuclei; BNC: binucleated lymphocyte cells; NDI: nuclear division index. **p < 0.01 vs vehicle (ANOVA + Dunnett's multiple comparison post-test).

(57%) protocols. The lack of a concentration−response relationship may indicate that, at the concentrations tested, **1** already reached the maximal effect attainable. Ethyl methanesulfonate is a monofunctional ethylating agent found to be mutagenic in a wide variety of genetic test systems, from viruses to mammals, and is known to be carcinogenic for mammals.[20] It has been reported to produce significant levels of alkylation in the bases, at the nitrogen and oxygen functionalities, by a mixed S_N1/S_N2 reaction mechanism.[17] According to Brookes and Lawley,[21] alkylation of guanine can cause point mutations, whereas depurination of alkylated guanine and subsequent chain scission can lead to segment mutations, which, in turn, cause chromosomal breaks and DNA alterations. In fact, ethyl methanesulfonate has been shown to cause major clastogenic effects in mammalian models.[16,17]

The protective effect of **1** against ethyl methanesulfonate appeared in both the pre- and co-treatment protocols, while no

Table 1. Effects of 1 on Frequency of Micronuclei (MN) and Nuclear Division Index (NDI) in Peripheral Blood Lymphocytes, following Ethyl Methanesulfonate and Colcemid Treatment (mean ± SEM, $n = 6$)[a]

	[μM]	pretreatment		co-treatment		post-treatment	
		MN/1000 BNC	NDI	MN/1000 BNC	NDI	MN/1000 BNC	NDI
ethyl methanesulfonate + 1	1930	14.00 ± 0.57[f]	1.53 ± 0.03	15.80 ± 1.16[f]	1.55 ± 0.04	20.14 ± 1.20[f]	1.41 ± 0.06
	0.3	5.50 ± 0.65[e]	1.46 ± 0.01	4.00 ± 1.23[e]	1.38 ± 0.01	19.25 ± 0.85	1.43 ± 0.01
	3.3	5.75 ± 0.48[e]	1.49 ± 0.01	6.75 ± 0.48[e]	1.58 ± 0.03	19.75 ± 1.25	1.36 ± 0.02
	33	3.75 ± 0.48[e]	1.42 ± 0.02	5.50 ± 0.29[e]	1.53 ± 0.04	19.50 ± 1.04	1.36 ± 0.01
	330	4.00 ± 0.58[e]	1.28 ± 0.01	4.50 ± 1.43[e]	1.63 ± 0.04	20.00 ± 1.29	1.31 ± 0.01
colcemid + 1	0.054	22.75 ± 0.94[f]	1.52 ± 0.03	24.78 ± 0.52[f]	1.39 ± 0.04	12.25 ± 0.85[f]	1.65 ± 0.08
	0.3	19.25 ± 1.70	1.44 ± 0.05	21.50 ± 2.22	1.37 ± 0.04	11.25 ± 1.20	1.59 ± 0.03
	3.3	18.42 ± 2.17	1.28 ± 0.03	21.25 ± 2.02	1.42 ± 0.04	12.25 ± 0.85	1.58 ± 0.05
	33	18.00 ± 2.04	1.31 ± 0.05	21.00 ± 1.83	1.51 ± 0.04	11.75 ± 1.05	1.56 ± 0.03
	330	ns	1.03 ± 0.01[g]	20.25 ± 1.38	1.41 ± 0.06	12.50 ± 1.05	1.56 ± 0.06
vehicle		3.33 ± 0.88[b]	1.63 ± 0.11	4.00 ± 0.41[c]	1.65 ± 0.06	3.33 ± 0.33[d]	1.59 ± 0.08

[a]For each treatment, MN frequency was determined by scoring at least 1000 and 500 binucleated lymphocyte cells (BNC). For untreated cultures, the value of MN/1000 BNC was 4.88 ± 0.13, while that of NDI was 1.54 ± 0.03. [b]EtOH 0.5% (24 h) and DMSO 0.5% (48 h). [c]EtOH 0.5% (48 h) and DMSO 0.5% (48 h). [d]DMSO 0.5% (24 h) and EtOH 0.5% (48 h). [e]Denotes significant differences from the mutagen ($p < 0.01$; Anova + Dunnett's multiple comparison post-test). [f]Denotes significant differences from the vehicle ($p < 0.01$; Anova + Dunnett's multiple comparison post-test). [g]Cytotoxicity occurred when an NDI value was ≤70% of control cultures and BNC were not scorable (ns).

effect was observed in the post-treatment mode. These three different experimental conditions are used to investigate the antimutagenic activity of a test substance. The pretreatment protocol allows evaluation of the capability to prevent mutagen-induced genotoxic damage. Co-treatment allows evaluation of the capability of the test compound to directly interfere with the mutagen to be studied, outside or inside the cell. Finally, the post-treatment protocol gives information about the capability of the test substance to repair mutagen-induced damage. Antimutagens acting in the pre- or co-treatment regimen are called desmutagens, while those acting in the post-treatment are named bioantimutagens.[23] Since the anticlasto-genic effect of 1 occurred as a result of the pre- and co-treatment, 1 behaved in the manner of a desmutagenic agent. Desmutagens are believed to act by inactivating mutagens, outside or inside the cell, before they can attack DNA.[22] Desmutagenic mechanisms include, for example, reducing and antioxidant effects, inhibition of the enzymes involved in the metabolic activation of the mutagen, alteration of the uptake, and chemical inactivation of the mutagen.

In regard to the specific mechanism of the desmutagenic activity observed for 1, an antioxidant effect can be excluded, because oxidative stress is not directly involved in the genotoxicity of ethyl methanesulfonate. Similarly, an interference by 1 with cytoplasmic enzymes may be excluded, considering that ethyl methanesulfonate is a direct-acting mutagen that does not require metabolic activation.[17] This test compound may alter the uptake of the mutagen by the cell membrane, as inferred from the anticlastogenic effect observed against ethyl methanesulfonate but not against colcemid. Thus, it can be hypothesized that 1 interferes in a specific way with the absorption of ethyl methanesulfonate. Unfortunately, this hypothesis is difficult to demonstrate because the mechanisms by which the two mutagens penetrate across the cell membrane are poorly understood. Early data showed that ethyl methanesulfonate is taken up into the embryos of barley by a passive process of diffusion,[24] while data on effects on human cells are not available in the literature. Similarly, data on colcemid absorption are not available. Considering the chemical structure of ethyl methanesulfonate, it could be hypothesized that this mutagen undergoes a chemical inactivation. As an

alkylating agent, ethyl methanesulfonate is able to replace a hydrogen atom in a molecule with an alkyl radical, by an electrophilic attack.[25] Under the present experimental conditions, ethyl methanesulfonate could react with 1 by inducing ethyl esterification of the carboxylic group and, in so doing, losing the ability to attack DNA. This reaction could be facilitated by both the buffered medium (pH 7.4), in which the carboxylic group is ionized, and the prolonged incubation time (24 h), during which time the two substances are able to come into contact. The chemical interaction between 1 and ethyl methanesulfonate may occur readily outside the cell under the co-treatment conditions, when the substances are simultaneously added to the medium. Under the pretreatment conditions, mutagen inactivation could occur inside the cell. In fact, when 1 is added to lymphocytes 24 h before ethyl methanesulfonate, it can enter into the cell, thus reacting with the mutagen in the cytoplasm after its absorption. In support of this hypothesis, some permeation kinetic experiments were carried out using multilamellar vesicles of dimyristoylphospha-tidylcholine as a biomembrane model and demonstrated that 1 is able to cross the phospholipid bilayers of the cell membrane and to reach the cytoplasm (Supporting Information).

On the basis of the present results, it can be hypothesized that the anticlastogenic activity of 1 is due to its ability to inactivate ethyl methanesulfonate by reacting with the mutagen outside the cell, in the medium, or inside the cell, after crossing the cell membrane. The antimutagenic activity here demonstrated suggests that 1 could be a useful chemopreventive agent.

■ EXPERIMENTAL SECTION

Lymphocyte Cultures, Solutions, and Chemicals. Peripheral blood cells were separated from whole blood obtained from healthy, nonsmoking males who were less than 40 years old and provided by AVIS (Italian Association of Voluntary Blood Donors). The donors provided written, informed consent for use of their samples. Lymphocytes were separated by using a density gradient (Histopaque 1077), then cultured in RPMI 1640 medium (2×10^6 cells in 5 mL) supplemented with 15% v/v fetal calf serum (FCS), 0.5% v/v phytohemagglutinin (2 mg·mL^{-1} in sterile deionized water), 1% v/v penicillin−streptomycin solution (5000 IU−5000 g·mL^{-1} in sterile deionized water), and 1% v/v L-glutamine (29 mg·mL^{-1} in sterile

deionized water). The cultures were incubated for 72 h at 37 °C in a moist (95% humidity), 5% CO_2 atmosphere.

3,4-Secoisopimara-4(18),7,15-trien-3-oic acid (**1**, MW = 302) as used in the present study was obtained by extraction from *Salvia cinnabarina* (96% HPLC purity grade), as previously described by Romussi et al.[12] The sodium salt of **1** was prepared by reaction with an equivalent quantity of NaOH (≥98% purity) in methanol (99.8% purity) and evaporation to dryness. To perform the experiments, the compound was dissolved in ethanol 50% v/v, then diluted in RPMI 1640 medium, in order to avoid its precipitation in the medium. The mutagens, ethyl methanesulfonate (≥98% purity) and colcemid (≥98% purity), were obtained from Sigma-Aldrich, Co. (St. Louis, MO, USA); all the other reagents, if not otherwise stated, were purchased also from Sigma-Aldrich. Ethyl methanesulfonate was dissolved in dimethyl sulfoxide (DMSO) (>99.5% purity) (Sigma-Aldrich Co., St. Louis, MO, USA); colcemid was purchased dissolved in Hank's buffered salt solution (10 mg·mL^{-1}) and added to cultures in this form.

Preliminary Assays. The cytotoxicity of **1** was evaluated initially to choose the test concentrations in the subsequent assays conducted. Cytotoxicity was evaluated by means of NDI, which is a measure of the effect on cell-cycle parameters.[26] NDI was determined by scoring at least 1000 cells for each concentration for the presence of one, two, three, or more nuclei and was calculated as follows: NDI = $(1M_1 + 2M_2 + 3M_3 + 4M_4)/n$, where $M_1 - M_4$ indicate the number of cells with 1−4 nuclei and n indicates the total number of cells scored. The percent NDI of treated cells (NDI$_t$) with respect to the control (NDI$_c$) was calculated as follows: NDI % = NDI$_t$/NDI$_c$ × 100. Cells that did not undergo mitosis, as judged by their size and the density of DNA-positive material, were not included in the count. The highest concentration at which neither necrosis nor cytotoxic or cytostatic effects were observed was used as the maximum concentration in the additional assays undertaken.

A further preliminary test was carried out to exclude a mutagenic effect of **1**. Mutagenicity testing was performed using the cytokinesis-block technique developed by Fenech and Morley,[27] in which cytochalasin B (Cyt B), an inhibitor of microfilament assembly, was added to cells to block the cytodieresis but not the nuclear division, so the cells become bi-, tri-, or multinucleated. Test solutions of the sodium salt of 3,4-secoisopimara-4(18),7,15-trien-3-oic acid were prepared by serial dilution (dilution factor 1:10) in RPMI 1640 medium. Sterile ethanol (50% v/v) was used as a negative control, while the positive controls were ethyl methanesulfonate (1.93 mM), an alkylating agent, and colcemid (0.054 μM), a microtubule-disrupting agent that binds tubulin. These concentrations induced a submaximal mutagenic effect (about 70%) and were obtained from the linear part of the concentration−response curve of mutagens. The experiments were repeated at least three times, and in each experiment, each concentration was tested in two parallel cultures; data obtained from at least two experiments were pooled in the statistical analysis.

After cultivation for 44 h, lymphocytes were supplemented with Cyt B (final concentration 6.25 μM) and incubated again for 4 h. At 48 h, cells were treated with **1**, at concentrations of 0.3, 3.3, 33, and 330 μM, and incubated again up to 72 h. At the end of the incubation period, the lymphocytes were collected, treated with a mild hypotonic solution (1:2 RPMI 1640 medium−H_2O, supplemented with 2% FCS) for 2 min, and then fixed in ice-cold acetic acid−methanol (1:1). After fixation, the cells were placed directly on slides, distributed by a cytospin centrifuge, air-dried, and stained with conventional May−Grünwald−Giemsa stain. All slides were coded and scored by a Zeiss Axioplan light microscope at 1000× magnification under oil immersion. NDI and MN were evaluated according to the criteria described by Fenech.[26] At each concentration, at least 1000 lymphocytes were scored to determine the NDI value, as described above, while at least 2000 binucleated (BNC) cells (1000 for each culture) were examined for the presence of micronuclei.

Antimutagenicity Assay. The test was performed by using the cytokinesis-block technique[27] slightly modified by Fimognari et al.[28] Solutions of the test compound were prepared as described in the preliminary assay section. Lymphocyte cultures were treated with the mutagens ethyl methanesulfonate (1.93 mM) and colcemid (0.054 μM), in the absence and presence of increasing concentrations of **1** (0.3, 3.3, 33, 330 μM); control cultures, treated with vehicle or **1** alone, were also included. The experiments were repeated at least twice, and for each experiment, each treatment was tested in two parallel cultures; data obtained were pooled in the statistical analysis. Three different treatment protocols were used to investigate the type of antigenotoxic activity of **1** (Figure 1).

The pretreatment window with **1** started at 24 h of culture and ended at 48 h. After incubation for 24 h, the cells were treated with **1** and incubated again. At 44 h, Cyt B was added to the cultures, and they were incubated again for 4 h. At 48 h, these were washed with RPMI 1640, treated with a genotoxic compound, and incubated up to 72 h. The co-treatment window with **1** started at 48 h of culture and ended at 72 h. After incubation for 44 h, Cyt B was added to the cultures, and these were incubated for a further 4 h. At 48 h, cells were washed with RPMI 1640, treated with both **1** and the genotoxic compound, and incubated again up to 72 h. The post-treatment window with **1** started at 48 h of culture and ended at 72 h. After incubation for 24 h, cells were treated with the genotoxic compound and incubated again for 20 h. At 44 h, the cultures were added with Cyt B and incubated again for 4 h. At 48 h, the lymphocytes then were washed with RPMI 1640, treated with the test compound, and incubated for 24 h. In each protocol, at 72 h the lymphocytes were collected, treated for 2 min with a mild hypotonic solution (1:2 RPMI 1640 medium−H_2O supplemented with 2% FCS), and fixed in ice-cold acetic acid−methanol (1:1). Slides were prepared, stained, coded, and scored, as described in the mutagenicity assay. Then, the NDI was determined and analysis of micronuclei was carried out.[26]

Statistical Analysis. All values are expressed as means ± SE. The one-way analysis of variance (one-way ANOVA), followed by Dunnett's multiple comparison post-test, was used to analyze the difference between treatments. A p value < 0.05 is considered statistically significant. Statistical analysis was performed with Graph-Pad Prism (Version 4.00) software (GraphPad Software, Inc., San Diego, CA, USA).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The anticlastogenic effect of the sodium salt of 3,4-secoisopimar-4(18),7,15-trien-3-oic acid (**1**) is related to its capability to be absorbed into cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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