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Anti-Inflammatory Senescence Actives 5203-L Molecule to Promote Healthy Aging and Prolongation of Lifespan

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ABSTRACT

The aging process depends on genetic stability, metabolic control, and resistance to stress; longevity in particular seems related to resistance to stress. Responses to stress anticipate adaptation to an unacceptable disparity between real or imagined personal experience and expectation, including adaptive stress, anxiety, and depression. However, if stress persists, it may lead to chronic diseases, ranging from inflammation and cancer to degenerative diseases. For some time, only remarkable stress was acknowledged to induce immune and vascular alterations, such as infection or hypertension. Now it is known that moderate stress independent of conventional risk factors can induce a potent alteration of health conditions and consequently shorten life quality and lifespan. Inflammation is a critical defense mechanism, that, uncontrolled, contributes to chronic conditions with inflammatory pathogenesis. Stressful life conditions turn out to induce a diffuse (systemic) pro-inflammatory status. Subclinical chronic inflammation is an important pathogenic factor in the development of metabolic syndrome, a cluster of common pathologies, including cardiovascular disease. Markers include mediators associated with endothelial activation and dysfunction. This work reports the *in vitro* and *in vivo* effects of the monoterpene AISA 5203-L on human vascular endothelial cells in reversing replicative senescence in preventing and alleviating nonpathological stress, as assessed by a functional observational battery (FOB) of 44 tests, addressing behavioral, neurological, and physiological criteria.

INTRODUCTION

INFLAMMATION IS ONE OF THE MOST ANCIENT defense mechanisms of the body and allows us to counteract environmental challenges. Following an aggression by trauma, light, or micro-organisms, inflammation takes place in every tissue; it is predominantly regulated at the vascular level. Responding to circulating pro-inflammatory cytokines, endothelial cells lining the vascular wall are able to recruit leukocytes into the injured tissue for immune survey following the expression of adhesion molecules. A significant part of the signaling of the response of the cell is due to the alteration of its actin cytoskeleton. Using human vascu-

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lar endothelial cells, we have shown that, out of hundreds tested, four molecules, which we have named *AISA* (anti-inflammatory senescence actives), were able to inhibit *in vitro* the expression of intercellular adhesion molecule-1 (ICAM-1), as well as other adhesion molecules, and the polymerization of actin fibers, following the activation by tumor necrosis factor-α (TNF-α). This cytoprotective capacity was maintained at all replicative passages of the cells. A patent has been filed thatprotects the criteria of selection and identification of the four AISA, as well as their therapeutic applications in pathologies associated with acute or chronic inflammatory stress.[1] In this work, we present the results of *in vivo* studies on one of them, (4R)-1-methyl-4-isopropenylcyclohex-1-ene (limonene), identified as AISA 5203-L (Fig. 1) administered *per os* at a concentration of 10 mg/kg to female rats put in a non-pathological stress situation.

Stress in response to an unacceptable disparity between reality and expectations leads to adaptation to stress, with anxiety and depression, and does not exclude stress persistence. Ongoing stressful life conditions may lead to chronic somatic complaints. For a long time, only extreme stress has been recognized to induce a propensity to infection or hypertension. Now it is known that even moderate stress, independent of conventional risk factors (dietary stress, smoking)—such as maternal separation, immobilization stresses, stressful life events in the middle aged and the elderly, academic stress—are all able to alter life quality and shorten lifespan by decreasing normal resistance.[2] Moreover, in humans, the outcome of stressful life conditions might be more complex than in animals, at least because of the role of language, written or spoken, and belief (religion, tradition, and other social structures that make stress tolerable or intolerable).

AISA 5203-L is a monoterpene. Terpenes are a large and varied class of organic components classified as secondary metabolites. They are produced by a wide variety of plants, particularly conifers, though also by some insects such as swallowtail butterflies, which emit terpenes from their osmeterium. They are the major components of resin, and of turpentine produced from resin. The name *terpene* is derived from the word *turpentine*. The smaller and more volatile terpenoids (C_{10} and C_{15}) are generally the main constituents of the essential oils obtained from many types of plants and flowers, widely used as natural flavor for food, as fragrances in perfumery, in aromatherapy, and in traditional and alternative medicines. Terpenoids possess a common structural feature: they contain an integral number of C_5 units (isoprene like) giving a basic molecular formula $(C_5H_8)n$ for the hydrocarbons series. They are derived from the metabolism of acetate by the mevalonic acid branch biosynthetic pathways of plants.

Examples of monoterpenes (C_{10}) are geraniol and limonene. Particularly, d-limonene has a pronounced chemotherapeutic activity and minimal toxicity in preclinical studies. A phase I clinical trial[3] performed to assess toxicity, maximum tolerated dose (MTD), and pharmacokinetics in patients with advanced cancer was followed by a limited phase II evaluation in breast cancer. Moreover, d-limonene is well tolerated in cancer patients at doses that may have clinical relevance.

In performing the experiments for assessment of the doses to be administered in an *in vivo* rodent model, an anti-stress effect of AISA 5203-L was unexpectedly revealed by a functional observation battery (FOB) based on 44 parameters addressing behavioral, physiological, and neurological parameters in female rats submitted to several stressful conditions.[4] Limonene and its metabolite perillyl alcohol have shown important effects leading to the capacity to tolerate stress and even pain when compared to vehicle-treated animals. Taken together, the *in vitro* results showing that AISA inhibit inflammatory markers irreversibly expressed in senescent cells[1] (Fig. 2B) made us presume that AISA would also interfere with the regulation, the extent, or the reversibility of the senescence process *in vivo*. The preliminary *in vivo* results reported here add evidence to the link between stressful conditions and vascular inflammatory reaction. As others do,[5] we consider endothelial dysfunction as the factual access to the generalized aging process. Finally, our work elucidates that AISA 5203-L effects may contribute to the regulation and the reversibility of the inflammatory response and thus its limitation.

MATERIALS AND METHODS

In vivo *chemistry studies*

Chemical characteristics of limonene: (4R)-1-methyl-4-isopropenylcyclohex-1-ene. Limonene ($C_{10}H_{16}$; CAS no. 5989-54-8; MW = 136.23) was selected out of hundreds of plant extracts obtained through bio-guided research HPLC, extraction, and purification in collaboration with the University of Montpellier and further screened on organ-specific endothelial cellular models (proprietary of AISA Therapeutics) for its anti-inflammatory properties (Fig. 1).

Drug preparation and assayed products. Limonene (R)-(+)-limonene), purity = 97%; and perillyl alcohol (S)-4-isopropenyl-1-cyclohexenylmethanol), MW = 152.23, purity = 98%.

In vitro *cell biology studies*

Endothelial cell culture. Human umbilical vein endothelial cells (HUVEC) were collected following normal deliveries from non-hypertensive, non-diabetic, non-smoking women, as we previously described.[6]

Limonene toxicity assays on HUVEC. For toxicity assays, we determined the effects of limonene on cell number variation by MTT assay and on cell proliferation (S phase determination) by BrdUrd incorporation. For cell number determination, HUVEC in 96-well culture plates, both at non-confluent and confluent distribution, were incubated with limonene at various concentrations for 24 h before performing MTT assay[7] (Fig. 2A).

For proliferation assays, non-confluent monolayers of cells cultured in 24-well plates were incubated with limonene at various concentrations for 24 h; 45 min before ending the culture, the cells were loaded with BrdUrd and treated with DNase I for 1 h at 37°C to expose incorporated BrdU. The cells were then labeled with fluorescent anti-BrdU antibody before detection by flow cytometry (FACSCalibur, Becton-Dickinson, Franklin Lakes, NJ), as described previously.[7] The percentage of cells in S phase was analyzed using CellQuest software. For these two tests, samples with proper concentration of DMSO were used as control.

Elisa of ECAM expression following HUVEC incubation with TNF-α. The detection of ECAM in endothelial cell culture after incubation with TNF-α was performed by use of the enzyme-linked immunosorbent assay (ELISA). Briefly, endothelial cells were grown to 80–90% confluence and treated for 24 h with TNF-α (0.2 ng/mL; R&D Systems Europe, Abingdon, United Kingdom) at 37°C. After stimulation with TNF-α, cells were washed with PBS, fixed in 2% formaldehyde for 10 min, then incubated with the primary anti-ICAM-I antibody (R&D Systems Europe) at 0.02 μg/mL for 2 h at laboratory temperature, and finally washed five times with PBS. Colorimetric revelation by mouse IgG1 isotypes linked with alkaline phosphatase (Chemicon, Euromedex, Temecula, CA) was performed, as described elsewhere.[8] The reaction was stopped after 20 min of incubation with NaOH 1M and the reading was performed at 405 nm (Fig. 2B).

Statistical analysis. Each experiment was performed in duplicate or triplicate and was repeated at least twice. The results for ELISA experiments are presented as mean as ± 95% confidence intervals of all of the values. A paired student's *t* test was used to evaluate statistically significant differences in ECAM protein levels between limonene, TNF-α-treated group, and control group, and between TNF-

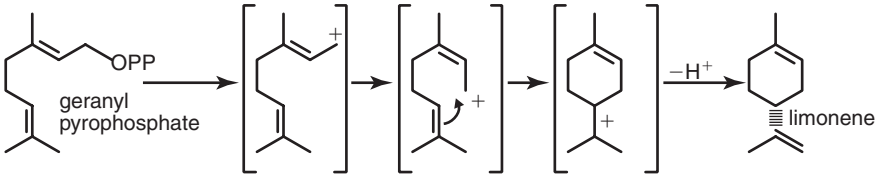

FIG. 1. AISA 5203-L (+)-limonene, purity = 97%.

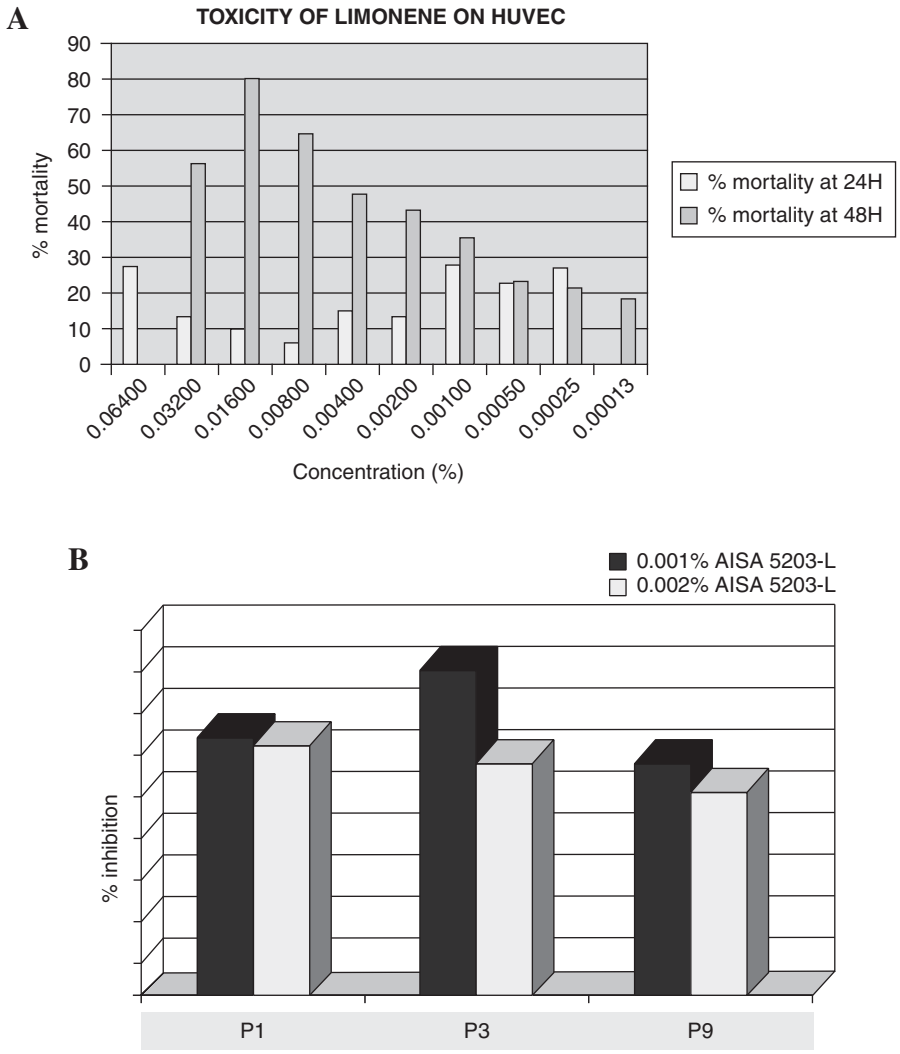

FIG. 2. *In vitro* results. (**A**) MTT toxicity test. (**B**) ICAM-1 inhibition at all PD (replicative senescence).

α-treated group and pre-treated with limonene group; $p < 0.05$ was selected as the statistically significant value.

In vivo *animal studies*

The *in vivo* studies were conducted according to the principals and guidelines of the ASAB, the Canadian Council for Animal Protection, and all the procedures were in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC of 24 November 1986, Official Journal L 358, 18/12/1986, pp. 0001–0028) on the approximation of laws, regulations, and administrative provisions of the member states regarding the protection of animals used for scientific purposes.

Functional observation battery (Figs. 3 and 4). Eighteen female rats Wistar HsdBrlHan (weighing 175–200 g) were obtained from Harlan France (Gannat, France). The 18 rats were weighed, marked, and divided into three experimental groups ($n = 6$):

- Control group: oral treatment with corn oil;
- Limonene 10 group: oral treatment with limonene at 10 mg/kg;
- Perillyl alcohol 10 group: oral treatment with perillyl alcohol at 10 mg/kg.

Measurement of limonene or perillyl alcohol in the FOB. Observations were carried out 60 min before treatment, and 60, 120, and 180 min after the oral administration of test substances

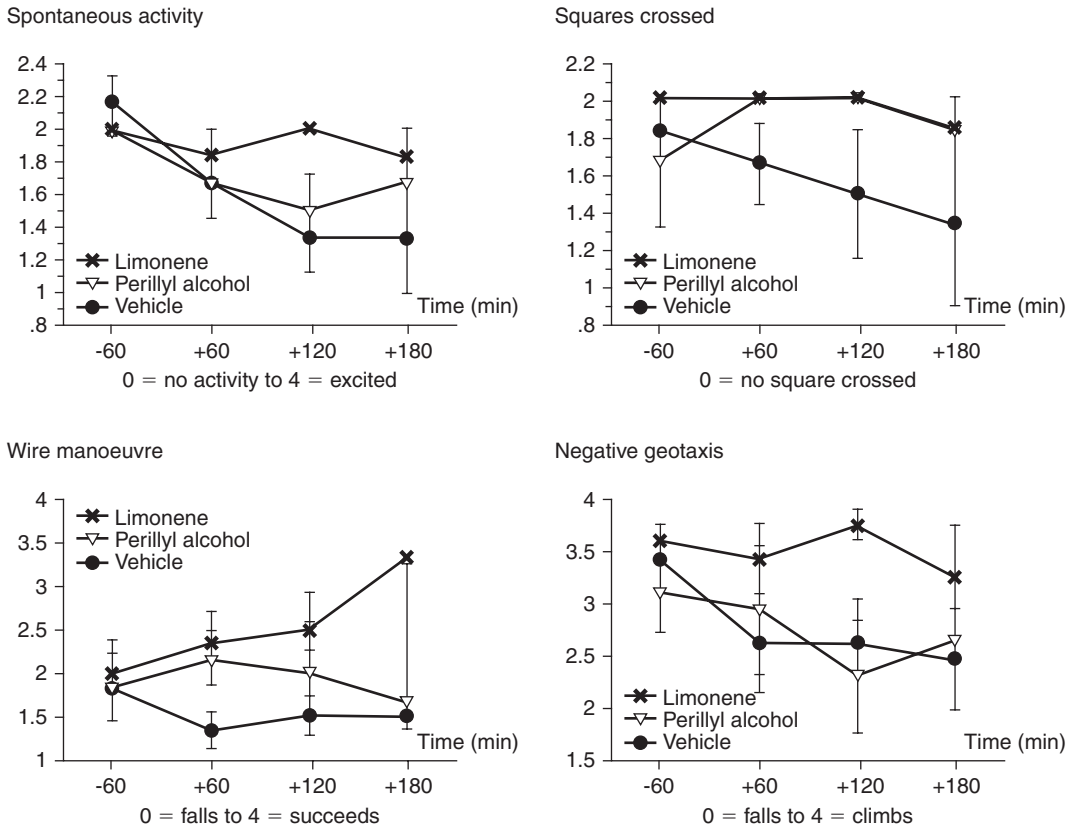

FIG. 3. *In vivo* results. Functional observation battery (FOB) results on motor function alterations following treatment with AISA-5203-L or its major metabolite perillyl alcohol.

with three observation phases: (1) a direct observation phase during which the animal was not disturbed; (2) an active observation phase during which the animal was manipulated; (3) a phase dedicated to the evaluation of the responses of the animals towards the reactivity assays.

The studied variables were the following:

- *Behavioral effects*: spontaneous locomotive activity, locomotive behavior troubles, anxiety, touch response, irritability, aggression and freezing caused behaviors, somnolence, number of defecations, number of miction, sensor-motor responses (toe pinch response and sound response);
- *Neurological effects*: pupillary reflex, palpebral closure, pelvic elevation, tail position, limb and abdominal tones, reversal test, grip test, tremors, and piloerection;
- *Physiological effects*: salivation, lacrimation, diarrhea, body temperature, respiratory rhythm.

Statistical analysis. Non-parametric tests were used. ANOVA using Kruskal-Wallis test was followed, when significant, by a Mann-Whitney test to compare the different studied variables to the vehicle-treated group. Differences were considered to be significant at the level of $p < 0.05$. All statistical analyses were carried out using the StatView5 statistical package (SAS Institute, Chicago, IL).

RESULTS

In vitro *studies*

Limonene toxicity assays. Figure 2A shows limonene toxicity at different concentrations on HUVEC. Toxicity is expressed as percentage of the mortality of cells at 24 and 48 h.

Effect of limonene on TNFα-induced ICAM-1 expression. Figure 2B shows the percentage inhibition of the expression of ICAM-1 by HUVEC at different replicative passages by

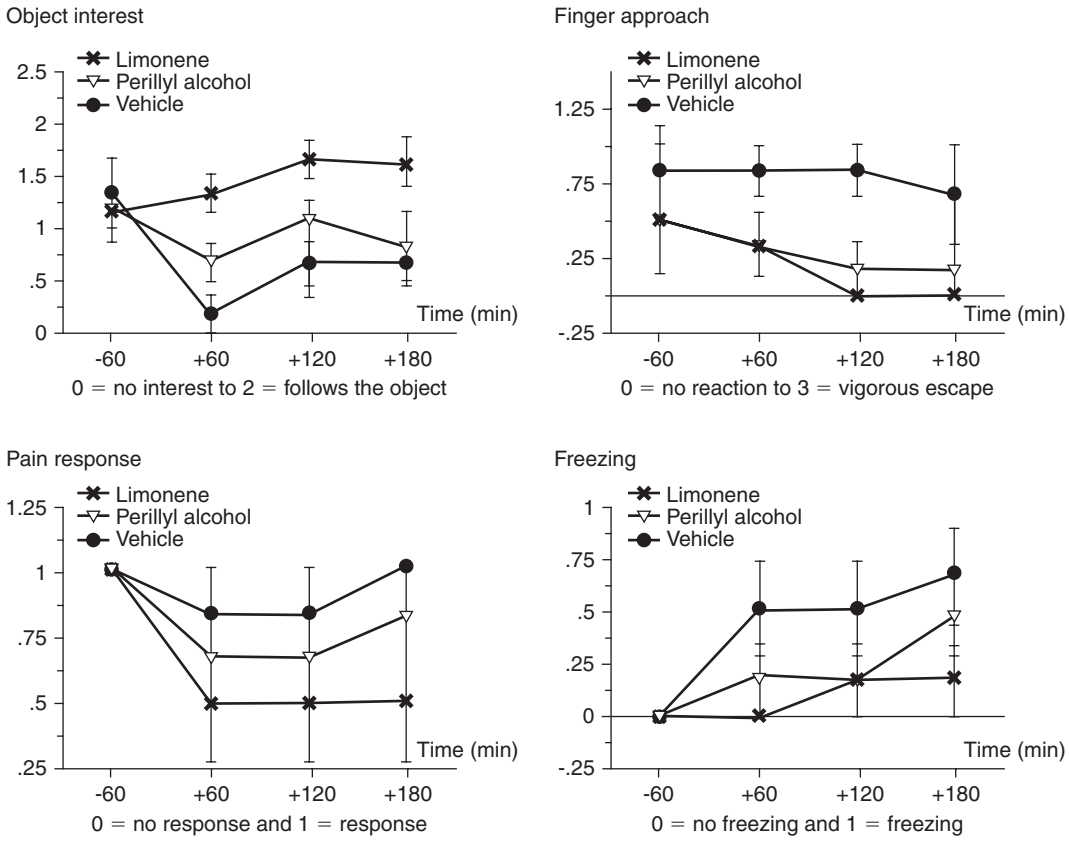

FIG. 4. *In vivo* results. FOB results on mood modulation following treatment with AISA-5203-L or its major metabolite perillyl alcohol.

limonene at 0.002% and 0.004% following incubation with TNF-α at a concentration of 10 ng/mL over 24 h.

In vivo *studies*

Determination of the doses by pharmacokinetic studies. The R-(+)-limonene was absorbed and rapidly metabolized,[9–11] its principle metabolites being the perillic and dihydroperillic acids[11] and perillyl alcohol.[12] In fact, 1 h following an oral absorption of labeled R-(+)-limonene (1 g/kg), the latter represented only 15% of the circulating radioactivity, 30% corresponding to the dihydroperillic acid and 50% to the perillic acid.[11] The oral absorption of the labeled product corresponds to a distribution into plentiful tissues,[10,11] followed by a principally urinary excretion without accumulation effects.[11] Limonene is also submitted to an enterohepatic re-absorption.[9,10] The oral bio-disponibility of R-(+)-limonene has been estimated at 43.0.[9]

The effective concentration determined by the cell biology studies of our *in vitro* screening platform was 100 μM,[1] thus representing approximately the concentration to be attained in plasma.[9,10] We have tested R-(+)-limonene *per os* in a model of female rats at the doses of 10 and 100 mg/kg in order to evaluate a dose-effect curve. The treatment consisted of a daily administration using corn oil as vector in consideration of the lipophilic character of limonene.

FOB results. The animals treated by limonene and perillyl alcohol in comparison with the control (vector) showed:

Significant changes in locomotive activity (Fig. 3)

- an increase in the number of squares crossed,
- an increase in the spontaneous activity,
- an improvement in the wire maneuver.

Significant changes in the reaction to pain and stress (Fig. 4)

- an increase in interest for an object, curiosity,
- less quick escapes after the finger approach,
- an increase in time before the rat falls from the wire,
- a decrease in toe pinch reflex,
- an important decrease in pain response,
- no aggression,
- no irritability,
- very poor audible vocalization,
- an important decrease in the startle response.

Significant physiological reactions

- a decrease in body temperature,
- an increase of the mictions.

DISCUSSION

Inflammation has become a modern plague. In a few decades, more than four generations of anti-inflammatory drugs have reached the market and have been adopted to treat all sorts of diseases based on inflammatory symptoms. Anti-inflammatory properties have been ascribed to dietary constituents also. For example, extra virgin olive oil containing oleocanthal,[13] a compound able to inhibit inflammatory pathways (COX-2 and COX-3), has been proven to be structurally close to ibuprofen. Biological roles of lactoferrin, an iron-binding glycoprotein found in exocrine secretions of mammals (breast milk, colostrum, tears, saliva, epithelial secretions, pancreas juice, bile) include antibacterial, antiviral, antitumor, and anti-inflammatory defense,[14] and a suppressive function towards various stressful life events correlated to the enhancement of opioid system directly and/or indirectly.[15]

d-limonene, a major component of citrus peel oil, but also found in breast milk and the prototype of monoterpenes in carcinogenesis studies (LD 50 = 5.3 g/kg in rats, oral administration), is formed by the cyclization of the 10-carbon intermediate geranylpyrophosphate (Fig. 1). d-limonene and its derived metabolites have been shown to possess cancer chemotherapeutic and chemopreventive efficacy in various preclinical models. Plasma metabolites of limonene are found in blood following ingestion of 100 mg/kg limonene and at least five compounds are present at 4 h after ingestion.[16] Although R- and S-limonene are only weak inhibitors of the isoprenylation enzymes, their major metabolites, perillic acid and perillyl alcohol, are more potent inhibitors (IC50 values in the low mM range). The metabolites possess greater activity towards the geranylgeranyltransferase type I enzyme than farnesyltransferase.[17] This is of particular relevance to us because, in the course of the vascular inflammatory response, endothelial cytoskeleton with its actin filaments and ICAM-1 interact through a signaling pathway, implicating proteins of the Rho GTPase family.[18,19] The activation of these proteins requires in fact a post-translational isoprenylation.

Again, the anticancer activities of geraniol (AISA 5202-G) and limonene (AISA 5203-L) seem to be linked in particular to an inhibition of the biosynthesis of isoprenoids and/or to the inhibition of transferases able to catalyse protein isoprenylation.[20] In consideration of the greater activity of its metabolites, limonene would play the role of a precursor.[21] Thus, the same mechanism of action described for the anticancer effects of geraniol or limonene could thus be at the origin of its anti-inflammatory characteristics. Indeed the capacity of geraniol to inhibit leukocyte adhesion induced by TNF-α has been recently reported.[22]

The clarification of the effects of AISA 5203-L on mood and behavior observed during a stressful situation, on neurological and motor parameters (Fig. 3), and especially its capacity to reverse stress to its opposite attitude, which is the interest for an object (Fig. 4), requires further studies. In fact our work aimed at the cytoprotection of activated endothelial cells[23] has allowed us to recognize that biological markers of inflammation are linked to those of stress and senescence. Moreover, the AISA mechanism of action may involve nitric oxide (NO), as shown for other drugs successfully treating vascular degeneration and senile dementia.[24]

Targeting vascular endothelium would allow to display concomitant anti-inflammatory, anti-stress and anti-senescence effects.[25]

The results on pain deserve a special consideration as it is an important aspect of the inflammatory response. Links between pain and inflammation have become evident with the discovery of opioid receptors expressed by lymphocytes (delta$_2$). Receptors μ3 have been described on human granulocytes and monocytes and on microglia and astrocytes,[26] and are coupled to the production of NO.[27] Vascular opioid receptors modulating the inflammatory signal transduction cascade permit us to hypothesize the potential link between stress and endothelial dysfunction. Individual's susceptibility toward pain may thus be a matter of stress, such as frequent irritability being a sign of becoming old. Briefly, after a decade or more of abundant literature on the concomitant emergency of inflammatory markers and carotid wall thickness or atherosclerosis, the relation between depressive symptoms and common carotid artery atherosclerosis has been finally described.[28]

In conclusion, AISA and other molecules of the sort have been recognized by man in the past and have been selected for their immune boosting and mood-modulating effects, as well as their anti-aging properties, as reputable by their presence in food, ritual objects, and toys.[29] Before now, Anthelme Brillat Savarin, defining *"gourmandize,"* said that "it makes the muscles stronger, and as the depression of the muscles causes wrinkles, those terrible enemies of beauty, it is true that other things being equal, those who know how to eat, are ten years younger than those ignorant of this science."[30] He then synthesized this statement into his famous aphorism: Tell me what kind of food you eat, and I will tell you what kind of man you are.

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