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Research article

d-Limonene challenging anti-inflammatory strategies

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Abstract: Aging and senescence seem linked by fundamental, yet still ill-understood mechanisms. For this reason, this paper expands on the background of a discovery that still has to gain acknowledgement by public policies to find its place in a market hungry for a non-toxic anti-inflammatory molecule. Reversibility of the senescent cell phenotype was the starting-point of a research that turned out to identify the monoterpenes class of molecules as able to achieve this goal. Indeed, these compounds strongly inhibit the circulation of pro-inflammatory cytokines as well as the expression of cell-anchored adhesive molecules, liable to recruit activated immune cells. Starting from cell-based studies, the pre-clinical and clinical assays reported here confirmed the capacities of these compounds, both in experimental colitis, dermatitis and stress murine models, but also in-human studies addressing the latent chronic inflammation associated with age or psoriasis. Last but not least, because of an intriguing mechanism yet not totally unraveled and most probably depending on the effect of monoterpenes on gut microbiota strains–apart from assuring a constant gut barrier repair-a consistent Quality of Life amelioration, i.e. mood modulation probably due to enhanced dopamine secretion was also demonstrated. Finally, after entering in more pharmacologic considerations on toxicity and bio-availability studies as for the safety of this class of compounds, a strategic positioning of the precious role of anti-inflammatory drugs in a market that has yet to overcome common chronic diseases because of their predisposing condition not only to cancer and neuro-degenerative diseases but now also to COVID-19 is envisioned.

Keywords: drug discovery; anti-inflammatory molecules; monoterpenes; *d*-Limonene; common chronic diseases; aging; cell senescence

1. Introduction

In an era of health standards reformulation inspired by the ambition of the population to improve Quality-of-Life (QoL), the assessment of new medical strategies has become a very important topic. Another recent trend is to rely more than ever on evidence-based medicine. For centuries, humans have intuitively looked in the vast array of plants surrounding them to develop safe yet efficient solutions to fight common illnesses. A scientific renewed interest for the healing properties of phyto-extracts emerged in the last decades of the XXth century. It has become more structured nowadays, fully respecting the principles of academic research and deeply interacting with the modern tissue of the pharmaceutical industry.

The most impressing story, very similar to the one reported here, is that of the discovery by an American team, in the 1980s, of the anticancer properties of a diterpene extracted from the bark of the Pacific yew (*Taxus brevifolia*), paclitaxel, marketed under the Taxol brand. In order to spare the trees, docetaxel as a diterpene was obtained by hemisynthesis in 1989 from a molecule extracted from the leaves of the European yew (*Taxus baccata*) at the Institute for the Chemistry of Natural Substances in Gif-sur-Yvette (France), then headed by Pierre Potier, to whom the French Centre National de la Recherche Scientifique (CNRS) gold medal will be awarded in 1992. The drug produced from the leaves was named taxotere in 1988 and was marketed in 1995 by Rhône-Poulenc Rorer. It is now marketed by Sanofi. From 1999 on, the royalties collected on the sales of this drug brought about 50 million euros per year to the CNRS. This is probably one of the most important actors of this revolution that emerged from the results of research in medicinal chemistry and addressed the issue of the conservation of precious plant elements to support the use of synthetic analogs.

In addition to great discoveries from plants, a certain awareness arose towards drug-related side effects, so that alternative research avenues had to be proposed for the most important issues of human health, such as common chronic diseases [1], resulting from chronic silent inflammation crossing the path of social inequalities [2].

In this article, a piece of this recent history of the search for pharmacological solutions to major health problems, whereby plant extracts found their way, is related. In particular, two contributions recently pointing to the particular discovery related in this paper are discussed. The first is the article of Alexey Moskalev of the Russian Academy of Sciences about “Terpenoids as geroprotectors” that appeared in 2020 [3]. The second is the publication by the Quintans group in Brazil, reporting a collaboration with the French CNRS, dealing with “Monoterpenes modulating cytokine” [4] Both these publications quote the pre-clinical and clinical work that we published and summarize here.

Of note, in the late 1990’s and early 2000’s, Cox-2 inhibitors cost the life of hundreds of thousands of patients [5,6] and it was mandatory to find less harmful drugs. The starting point to search for such an alternative non-toxic anti-inflammatory “lead” inspired a bio-guided research and was based on cellular criteria, namely the definition of new targets to dampen the inflammatory process. It included novel biophysical parameters such as evaluation of the tensional modifications of the cytoskeleton associated to stress and inflammation [7,8].

The outcome of the long research that will be related in detail here, was covering for the first time a new yet still unnamed aspect of inflammation, the physical dynamics of morphogenesis in cells undergoing senescence. Once senescent, cells indeed become a source of additional pro-inflammatory signals in the body that can be inhibited by glucocorticoids [9]. In this context**,** new

results demonstrated the potent inhibitory role of the monoterpene *d*-Limonene. Apart from being known in cancer research [10,11] as an inhibitor of iso-prenylation of Rho, Rac and Ras, *d*-Limonene turned out to be able, following TNF-α stimulation, to inhibit more than 80% of the expression of the transcription factor NF-κB [12], giving rise to a new perspective in anti-inflammatory drug discovery.

Yet, as will be shown at the end of this review, also largely covering characteristics of biologics [13] and glucocorticoids [9], last but not least, the Covid-19-related "cytokine storm" could be contained by old [14] and new drugs [15].

2. The historical perspective

Limonene is a chiral monoterpene, naturally present as two isomers, *d*- and *l*-Limonene (Its official name by the International Union of Pure and Applied Chemistry (IUPAC) is 1-Methyl-4-(1-methylethenyl)-cyclohexene.) [16,17]. The dextrogyre form, *d*-Limonene, is the most abundant in nature and widely used for its properties as a potent solvent. It is a transparent-to-yellow liquid, with a distinct orange smell. The levogyre *l*-Limonene is scarcer and has a piney odor.

In nature, *d*-Limonene is present in many plants and, of note, is very abundant in the peel of citrus fruits [17]. Essential oils derived from these peels have long been used as food additives, because of their flavor and preservative properties. Their medicinal properties have also long been recognized, especially as antiseptics and wound healing compounds [17,18]. The soothing effect of orange peel extracts was also identified early [19].

The most recent history in the revival of *d*-Limonene, as a nutraceutic with pharmacologically interesting properties, was initiated at the end of the XXth century. At that time, a search was conducted to find new non-toxic anti-inflammatory compounds. Indeed, as mentioned above, the medical world was just recovering from the unfortunate cardiovascular side-effects of inducible cyclo-oxygenase 2 (Cox-2) inhibitors [5,6]. That these compounds had been responsible for thousands of cardiac infarcts raised the question of a model used to screen potential new molecules. A novel possible route was to investigate for activities directed at restoring the homeostasis of inflamed endothelial cells [20]. Upon inflammation, the latter undergo a modification of their cytoskeleton and express adhesion molecules, a mechanism reversible after resolution of the inflammatory episode. However, aging endothelial cells loose this capacity of reversibility, i.e. to shift their cytoskeleton from a tense to a relaxed mode [21,22]. Consequently, they become unable to re-internalize adhesion molecules of the CAM (Cell Adhesion Molecules of the immunoglobulin superfamily) family (ICAM-1, VCAM-1) or selectins (E-selectin, P-selectin). Since the latter interact with ligands on immune cells (notably integrins), endothelial cells thus keep attracting pro-inflammatory leukocytes such as neutrophils and consequently favor the chronic inflammation associated with aging. As was shown later by the team of Judith Campisi [23,24], these aged endothelial cells are also capable of secretory activities, thus entertaining a pro-inflammatory loop, which is the hallmark of senescence. The molecular mechanisms of inflammaging thus comprise complex interactions between adhesion molecules and pro-inflammatory cytokines as well as an interplay between senescent vascular cells and innate immunity partners such as neutrophils and monocytes/macrophages (Figure 1).

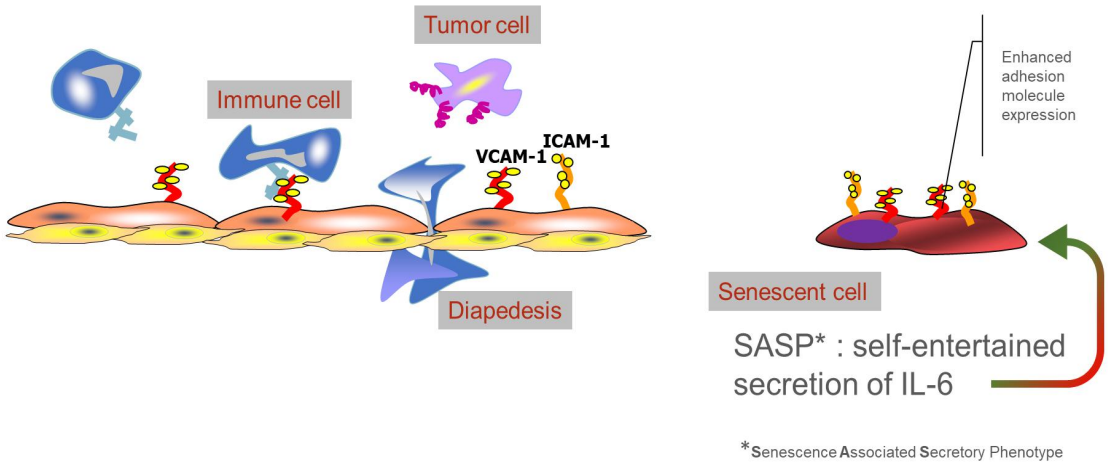

Figure 1. Similarities between inflammation (left side) aiming at recruiting neutrophils and macrophages *vs* senescence (right side), where these mechanisms become self-entertained independently from environmental challenge.

3. Cell-based experimental models of inflammation and aging

In order to search for compounds able to counter cell stress and the persistence of adhesion molecules expression *in vitro*, a model had been previously published that could readily be applied [25].

Primary organo-specific endothelial cell cultures were established from fresh samples and passaged whenever they reached confluence [26]. Passages were stopped when cell senescence became obvious, based on cell shape (assessed by microscopy), cytoskeleton changes and expression of adhesion molecules. For the latter two, confocal microscopy was used. Cells were permeabilized and stained using rhodamine-conjugated phalloidin binding the cytoskeletal molecule actin, allowing to detect whether it was polymerized or not. FITC (fluorescein isothiocyanate)-conjugated monoclonal antibodies were applied to unmanipulated cultures to assess the level of surface expression of abovementioned adhesion molecules.

This model was applied to a large array of primary cultured endothelial cells. Young animals (mice, rats, rabbits, guinea-pigs, calves…) were used to obtain fresh cells to be "aged" *in vitro* by a series of passages on Petri dishes. Spontaneously aged endothelial cells were also obtained from a series of older animals (ornithorynx, monkey, donkey, parrot, duck, zebra, lion…). For human cells, umbilical cord endothelial cells (HUVEC) as well as primary cultures of endothelial cells from different organs (liver, kidney, spleen, uterus, heart, left and right auricles, brain…) were studied.

The extensive species- and organ-related cell-set of this model was designed to provide precise information on both anti-inflammatory and anti-senescence properties of the molecules tested. Indeed, replicative senescence has a species-specific course illustrated by the fact that "aged" endothelial cells can be obtained *in vitro* from mouse endothelium after 12 passages, while it takes 96 passages for bovine endothelial cells [27]. Endothelial cells from "old" animals spontaneously

display the same characteristics as cells aged *in vitro*. Similarly, organ-specific senescence could be demonstrated by studying human endothelial cells from different organs.

Besides spontaneous or passage-induced senescence, cultured endothelial cells were challenged by pro-inflammatory cytokines (TNF-α, IL-6, IL-1β, IFN-γ…), reactive oxygen species (H_2O_2) or pro-oxidants (vitamin C, glucose...). Such challenges were followed by the same examinations by confocal microscopy of cells grown on coverslips. This investigation was completed by Elisa determinations of cytokine production by the same cells cultured in small wells as a validation assay.

These preliminary studies allowed to demonstrate that inflammatory stimuli could mimic in young cells the effects of senescence on the cytoskeleton and on the expression pattern of adhesion molecules, while only minor modifications were induced on aged cells. However, once the stimulus was removed, stressed young cells were able to return to a "pre-stressed" state of a "hairy-like" actin meshwork (within quiescent cells actin fibers are disposed in an entanglement of loose and fine fibers; upon stimulation by various drugs toxins or pro-inflammatory cytokines, the actin polymerization is giving rise to structured uni-directional cables called "stress fibers") and low adhesion molecules expression, while no change could be seen in senescent cells.

Putative anti-inflammatory drugs could thus be tested on both "young" and "aged" cells as well as on "resting" and "inflamed" cultures obtained from different species and organs.

This particular approach was largely inspired by the work of Gerald Edelman [28] and Donald Ingber [29,30]. The latter had attributed a pivotal role in cellular signal transmission to the tension sustained by different states of actin polymerization justifying a more or less stretched actin cytoskeleton.

Indeed, in endothelial cells, actin stress fibers, obtained by intense actin polymerization following pro-inflammatory cytokines stimulation, induce ICAM-1 and VCAM-1 expression on the cells' surface [22]. This enhanced expression of adhesion molecules is the pre-requisite for leukocyte recruitment by the activated endothelium for enhanced diapedesis as mentioned above [21]. Senescent endothelial cells maintain an overexpression of adhesion molecules at their surface, because of their impossibility to reverse the tensional state of the stretch fibers from tensed to randomly diffuse.

Overall, there is thus a great similarity between a newly inflamed endothelial cell and a senescent endothelial cell, apart from their different morphology. The crucial difference, however, is that modifications of a young inflamed cell are totally reversible once the inflammatory stimulus has been removed while a senescent cell can no longer return to a resting state. As mentioned above, this maintains leukocyte recruitment and entertains chronic local inflammation accelerating aging.

4. Origin of *d*-Limonene: eastern healing plants

Local tradition, as previously pointed out, has long used various parts of plants, and notably plant extracts, in numerous healing preparations [17]. Thanks to the collaboration with Pierre Potier, the aforementioned discoverer of taxotere, an original and exceptional collection of plant extracts from the Hanoi University was granted to our research group, settled at the time at the Faculty of Medicine of the Necker-Enfants Malades Hospital in Paris (University Paris 5 René Descartes), by the "Institut de Chimie des Substances Naturelles" (Gif sur Yvette, France). Indeed, the University of Hanoi prepared every year plant extracts from a number of specimens collected by ethnobotanists at the frontier between China and Viet-Nam (personal communication by Pierre Potier), guided by

instructions from local medicine-men about their properties of wound-healing, antipyretics, or appeasing the inflammation and pain caused by mosquito bites. A considerable number of such complex plant extracts [31] were studied and analysed in collaboration with Chantal Menut, professor of organic chemistry at the University of Montpellier, a unique specialist of natural volatile extracts, trained in Africa, South America and New Caledonia. The most abundant components, identified in these extracts by gas chromatography-mass spectrometry (GC-MS), were then selected for further biological tests.

For each of these compounds, tested at various concentrations, the inflammation-induced models described above were applied after a pre-incubation of 1h, 16h or 24h with the putative active molecules, or after stressing the cells, or directly on aged cells [32].

At the end of this extensive screen, four molecules were finally identified with the expected activity on all cell types (organ-derived) and in all animal species tested. They were respectively: geranyl acetate, geraniol, *d*-Limonene and isomenthone. Notably, *d*-Limonene was present in the tested extract of *Halfordia kendack (Rutaceae)*. This tree, initially discovered in New Caledonia, has a natural habitat in Australasia and in tropical and subtropical rainforests [33]. In fact, *d*-Limonene is also present in numerous species of the *Citrus* genus that belongs also to the *Rutaceae* family.
Oranges (*Citrus sinensis* L) are safe to obtain, compared to the first exotic plants holding the rare terpenoid tested. Last but not least, this choice was also driven by the necessity to protect rare Asian plants. Further studies were therefore conducted with orange peel extract (OPE) obtained by cold pressure from organic oranges checked for the absence of pesticides or other potential organic or chemical contaminants (herbicides in particular as they may display neurotoxic activities). Indeed, OPE contains large quantities of *d*-Limonene (up to 95%).

The exceptional properties of *d*-Limonene metabolite perillyl alcohol (POH) were later uncovered (Perillyl alcohol derives its name from another plant with medicinal properties, *Perilla frutescens*, reported as early as by Pliny the elder for its use by patricians in Rome to delay aging.). Both *d*-Limonene and POH displayed in all tested species and in all submitted conditions, an anti-inflammatory effect based on 80% inhibition of adhesion molecules expression and disruption of actin stress fibers that had appeared upon stimulation of endothelial cells with pro-inflammatory agents. Moreover, when applied to aged cells (from old animals or after *in vitro* passages), they were able to reverse these senescent characteristics in a great number of species and cell-types. Of note, only these four compounds, out of 2000 tested, displayed the broad activity investigated for, i.e. whatever the organ and species used to derive primary endothelial cell cultures.

These results were used to apply and obtain a first patent [34]. The latter describes how these four molecules were discovered and covers all precursors or metabolites of *d*-Limonene. Moreover, it claims the activity of these molecules to inhibit not only the recruitment of leukocytes in inflammation but also that of metastatic cancer cells, based on mechanisms involving the same adhesion molecules.

During the same period of our investigation, *d*-Limonene, isolated from a chemical library, was characterized for its action on Rho isoprenylation [10,11]. This work, conducted by Pamela Crowell at Wisconsin University (USA), focused on the Rho-Rac-Ras pathway in oncology and also demonstrated a role for POH on Ras farnesylation as an anti-tumor mechanism in pancreatic cells [35]. These studies, subsequently published, were supporting the therapeutic properties of this monoterpene for the treatment of pancreas, colon or breast cancer [36].

5. Mechanism of action of *d*-Limonene, *in vitro* activity

In a famous paper of the early 2000, the Belgian researcher Olivier Toussaint dubbed the acceleration of fibroblasts replicative senescence observed under stress conditions "Stress Induced Premature Senescence (SIPS)" [37]. At about the same time the Italian immunologist Claudio Franceschi officially introduced the term "inflammaging" [38], identifying chronic inflammation as favoring senescence. To complete this picture of stress accelerating cell senescence associated to clinical chronic diseases, around that period also emerged the notion of « silent inflammation » considered to be the basis of such dreadful clinical conditions as cancer, degenerative and chronic inflammatory diseases, as well as possibly metabolic syndromes, including diabetes and obesity.

Yet, at the beginning of the 1990's already [28] the Nobel prize winner and immunologist Gerald Edelman had identified the functions of cell adhesion molecules as strategic in linking immune functions, aging and cancer. He had done more, introducing a sort of dynamical vision of biology, a concept derived from mathematics. Combinatorial degeneracy relates indeed to the versatility of relationships between CAMs and integrins, as mentioned earlier. This touches an immense chapter in cell biology, namely signal mechano-transduction primarily, as highlighted by the work of Donald Ingber.

At the end of the 1980's / beginning of the 1990's, this scholar of Judah Folkman focused on an intrinsic yet overseen aspect of cell functioning, i.e. cells' capacity to develop a force, thanks to the modifications of their elastic properties. This approach had led to coin the term of signal "mechano-transduction" [39], because most of cell-signaling activities clearly depend from their tensional status. The latter was identified to reside in modifications of the actin and myosin cytoskeleton, sustained by the micro-tubular system.

These different approaches are really at the basis of the discoveries reported here on identifying a new anti-inflammatory non-toxic agent. Indeed, these various aspects of cell behavior, inflammation and aging are linked. As mentioned briefly above, a pro-inflammatory cytokinic environment is liable to activate peripheral immune cells. This activation involves polymerization of the actin cytoskeleton in both immune and endothelial cells. In turn, integrins and CAMs are upregulated, allowing for the recruitment of circulating cells *via* diapedesis. If these mechanisms persist, for instance in stressing conditions, an acceleration of the steps leading to the replicative cell senescence process in the cell occurs, eventually leading to its dysfunction [32].

An important consequence of the recognition of the mechano-transductional pathway is that pro-inflammatory signaling can be inhibited by counteracting the formation of actin stress fibers in the cell. This is due to the enhanced pathways leading to nuclear transcription of cell adhesion molecules upon an increased intracellular tensional status. This model has been largely exploited for cancer studies connecting extracellular matrix-driven and cytokine stimulations [29].

On this background, several *in vitro* experiments were conducted in an attempt to decipher the mechanisms leading to actin disruption and down-modulation of adhesion molecules upon exposure to *d*-Limonene, in endothelial cells.

6. Endothelial cell cytoskeleton as a tool for drug discovery

A model of vascular microtubules formation on matrigel was designed, based on human microvasculature endothelial cells (HMEC) and human bone marrow endothelial cells

(HBMEC) [40]. A strong inhibition of tubule formation was observed in the presence of POH at 125 and 250 mg/mL and a slightly lesser effect for *d*-Limonene, only inhibiting microtubule formation at the highest dosage of 250 mg/mL. This suggested that besides decreasing the expression of adhesion molecules on endothelial cells, monoterpenes can also act directly on angiogenesis, thereby limiting the spreading of inflammation and potentially tumor growth and/or metastasis [40].

Because inflammation and angiogenesis rely on TNFα-induced translocation of NF-κB, a model was set-up to evaluate the role of *d*-Limonene and its metabolites on the NF-κB pathway. Fibroblast cultures were challenged *in vitro* by exposure to TNF-α and NF-κB induction was measured by Western blot [12]. TNF-α, with or without OPE, did not alter cell viability as shown by consistent levels of γ-tubulin and β-actin, indicating the absence of reduction in the number of cells pelleted prior to Western blotting. DMSO alone (vehicle) did not induce any translocation of NF-κB while the latter was obvious with cannabis-based drugs or isopentenyladenosine. TNF-α similarly induced a significant translocation of NF-κB, that was decreased over four-fold in the presence of OPE.

In a third model, related to the experiments on colitis described below, the tightness of intercellular junctions was tested [12]. The hypothesis was that the integrity of cell-junctions could be contributing in a relevant way to the modulation of intracellular tension and thus of pro-inflammatory gene regulation. *d*-Limonene was added on cells from the colonic cell line HT-29/B6 cultured in monolayers. Epithelial resistance was measured and shown to increase significantly in the presence of OPE or POH. *d*-Limonene could thus act in limiting diapedesis also by strengthening cell junctions and might be efficient as well in maintaining or strengthening the epithelial gut barrier.

This particular aspect now related to as “barrier dysfunction repair” or “intestinal barrier protection” will gain an immense importance in the following years. In fact, it was not known at the time that the breach in enterocytes tightness could be provoked by many conditions [41], such as pregnancy and menopause, allergens and toxins, as well as sugar and its end-products, but most of all by prolonged endogenously produced cortisol or by corticoid treatment, when neither NSAID nor biologics would work.

7. Pre-clinical and clinical trials : health allegations of a novel nutraceutical

As described above, *d*-Limonene had been selected for its extensive anti-inflammatory and correlative anti-senescence properties. The latter had so far been demonstrated by the inhibition or reversion of the expression of adhesion molecules and actin polymerization in primary endothelial cell cultures. The proof of concept had then to be tested *in vivo.* To this avail, animal models were developed, respectively of colitis and dermatitis, later followed by first-in-man trials.

7.1. d-Limonene as a nutraceutic

7.1.1. Rat colitis model

Since our experimental data had demonstrated that *d*-Limonene is able to strengthen the barrier function of enterocytes, an animal model of colitis was designed to test the capacity of orally administered *d*-Limonene to exert this activity *in vivo* together with its anti-inflammatory properties.

In this study [12], a rat model of TNBS (2,5,6-trinitrobenzene sulfonic acid)-induced colitis was developed in three batches of animals which were compared to control unmanipulated rats. Among

animals with TNBS-induced colitis, one lot received ibuprofen as anti-inflammatory drug and one was orally fed with *d*-Limonene. Peripheral levels of TNF-α were assessed in all animals. At the end of the experiment, the animal's colon morphology and pathological aspects were examined and scored according to published criteria.

As expected, a significant level of inflammation, resulting in gut morphological alteration, pathology-assessed inflammatory lesions and increased levels of peripheral TNF-α, developed in untreated rats with TNBS-induced colitis compared to unmanipulated animals. All inflammatory scores were significantly reduced by ibuprofen, also as expected. Yet, *d*-Limonene feeding resulted in a similar reduction of all scores of colon morphological alteration and tissue inflammation, and was accompanied by a significant inhibition of the TNBS-induced increase of peripheral TNF-α levels.

This model confirmed the potent anti-inflammatory properties of the non-toxic, plant-extracted, *d*-Limonene, in line with the initial hypotheses of identifying an effective nutraceutic. Indeed, the clinical course of TNBS-colitis induced rats fed *d*-Limonene suggests a beneficial role of *d*-Limonene as a diet supplement impairing inflammation.

7.1.2. Rat stress model

During the pre-clinical studies reported above, ethologists conducting the study (ETAP in Nancy, France) also observed behavioral changes in treated animals. These observations led to an additional pre-clinical study on stress in rodents [42]. A functional observational battery (FOB) was set up and applied to various conditions of stress, i.e. imbalance, light and sound stimulation or pinching. The FOB confirmed the stressed status of control vehicle-fed rats. Conversely, a series of parameters were significantly less disturbed in rats fed *d*-Limonene who retained a better activity and displayed less signs of stress. These effects were more pronounced and sustained after ingestion of *d*-Limonene than of POH, suggesting the role of endogenous metabolization of the terpene.

Stress is closely linked to inflammation by its biological mechanisms and its consequences on accelerated aging [43]. As stress triggers a hormonal response along the hypothalamus–pituitary–adrenal (HPA) axis, it can disrupt the ortho/parasympathetic balance. According to the improved FOB of treated rats, *d*-Limonene was able to alter ortho/parasympathetic parameters as well as central neurotransmitter functions. This study showed that *d*-Limonene exerts a significant anti-stress action measurable by behavioral and physiologic parameters under the influence of the nervous system. In addition to its anti-inflammatory effects, a beneficial role as an anti-stress substance could thus also be claimed for *d*-Limonene used as a dietary supplement.

This is also sustained by an independent Japanese study demonstrating that *d*-Limonene is able to induce an increase of catecholamines and notably dopamine [44].

7.1.3. The FP7 RISTOMED project, first in-human study

Eating habits may influence the life span and the quality of aging process by modulating, among others, the inflammatory state and the onset of chronic inflammatory diseases. The multi-centric, multi-national RISTOMED project was developed to provide a personalized and balanced diet, enriched or not of nutraceutical compounds, to decrease and prevent inflammaging, oxidative stress and gut microbiota alteration in healthy elderly people [45]. The research focused on the effect on

inflammation and metabolic markers after 56 days of RISTOMED diet alone or supplementation with three different nutraceutical compounds, i.e. a probiotic blend, OPE containing known amounts of *d*-Limonene given twice daily as soft gel capsules or argan oil.

A cohort of 125 healthy elderly subjects was recruited and randomized into 4 arms : Arm A, RISTOMED diet; Arm B, RISTOMED diet plus the VSL#3 probiotic blend; Arm C, RISTOMED diet plus AISA *d*-Limonene; Arm D, RISTOMED diet plus Argan oil. Inflammation and metabolic parameters, as well as the ratio between *Clostridium* cluster IV and *Bifidobacteria* (CL/B) were collected before and after 56 days of dietary intervention, and their evolution compared among the arms. Moreover, participants were subdivided according to their baseline inflammatory parameters (erythrocyte sedimentation rate [ESR], C-Reactive Protein, fibrinogen, TNF-α, and IL-6) in two clusters respectively with low or medium/high level of inflammation. The evolution of the measured parameters was then examined separately in each cluster.

Overall, RISTOMED diet alone or with each nutraceutical supplementation significantly decreased ESR. RISTOMED diet supplemented with *d*-Limonene resulted in a decrease in the levels of fibrinogen, glucose, insulin and IL-6 together with a lower HOMA-IR (Homeostasis Model Assessment of Insulin Resistance). The most beneficial effects were observed in subjects with a medium/high baseline inflammatory status who received RISTOMED diet with *d*-Limonene supplementation [45].

This study emphasizes the beneficial anti-inflammaging effect of RISTOMED diet supplemented with nutraceuticals such as OPE to control the inflammatory status of elderly individuals.

Of interest, and in line with the anti-stress effects in the FOB rat model reported above, RISTOMED volunteers were asked to answer standardized questionnaires investigating for mood and well-being (anxiety, depression and grip strength). Only OPE supplementation demonstrated a significant and positive difference after 56 days of controlled diet [45].

7.2. d-Limonene as a topic anti-inflammatory addressing auto-immune diseases

Psoriasis and atopic dermatitis are two conditions of chronic skin inflammation for which a number of therapeutic approaches have been tested with disputable success. Based on the anti-inflammatory and anti-angiogenic properties of *d*-Limonene reported above, two murine models were designed to assess the potential benefit of this molecule on skin lesions [40].

7.2.1. Dermatitis murine model

Dermatitis was developed in three batches of mice by application of 12-O-Tetradecanoylphorbol-13-Acetate (TPA), and compared to unmanipulated animals [40]. In one batch, the lesions induced were untreated, while the two other groups of mice were treated by topical application of *d*-Limonene or POH. At the end of the experiments, macroscopic and microscopic evaluation of skin lesions was performed. P-selectin expression was assessed in immunohistochemistry on skin biopsies, and serum concentrations of IL-1β, IL-6 and TNF-α were measured. Both *d*-Limonene and POH reduced the severity and extension of TPA-induced skin lesions with significantly lowered macroscopic and microscopic scores. Moreover, the expression of P-selectin induced by TPA was abrogated by POH and significantly lower serum concentrations of

IL-6 and TNF-α were observed in *d*-Limonene- and POH-treated mice.

7.2.2. Wound healing mouse model

In this model a mechanical skin lesion (incision) was used to assess the efficacy of *d*-Limonene and POH applied topically in two batches of mice compared to one batch left to heal spontaneously. All animals successfully healed, but faster after application of *d*-Limonene or POH [40]. At the end of the experiment, the healed skin was examined for angiogenesis. The neo-angiogenesis associated to tissue regeneration was extended as expected in spontaneous healing. However, it was significantly lower in treated animals with clearly reduced neovascularization. Moreover, the level of expression of P-selectin on skin blood vessels was significantly reduced in healed animals treated with *d*-Limonene and POH.

These studies show that *d*-Limonene and POH demonstrate significant anti-inflammatory effects in murine dermal inflammation and wound-healing. The decreased systemic cytokine production, as well as a consistent inhibition of endothelial P-selectin expression and neo-vascularization induced by these terpenic compounds contribute to their healing effects on the epidermal barrier. This led to the development of an observational study in psoriasis patients [46].

7.2.3. Intermediate psoriasis, in human observational study

An open non-randomized setting enrolled nine patients with moderate psoriasis. OPE containing known amounts of *d*-Limonene were given twice daily as soft gel capsules ingested and/or emptied and massaged on skin lesions, for 45 days. In spite of the small size of the cohort, highly significant positive effects were observed, with a reduction of clinical scores such as Psoriasis Area Severity Index (PASI) and Videodermoscopy Scalp Psoriasis Severity Index (VSPSI). Moreover, on top of the global subjective satisfaction of the patients, a significant improvement of the objective Dermatology Life Quality Index was also recorded [46]. This encouraging observational study should serve to prompt a larger randomized blind study. Indeed, implementation of a non-toxic substance of natural origin in the widespread condition of psoriasis could represent a significant advance in the treatment of this disease.

8. *d*-Limonene pharmacokinetic and toxicity studies

In order to better understand how triggering an inhibition of the pro-inflammatory signal cascade is impacting the mechanistic functioning of several bodily functions, a brief complement about pharmacokinetics, bio-availability and toxicity studies of *d*-Limonene is deserved.

d-Limonene [(4R)-1-methyl-4-isopropenylcyclohex-1-ene (CAS: 5989-27-5), MW 136,24 g/mol] is a major component of orange peel. This prototype of cyclic monoterpenes is biosynthesized from the C10 precursor of monoterpenes, geranyl diphosphate (GDP); the conversion of GDP to limonene involves both isomerization and cyclisation to get the α-terpinyl cation from which Limonene is obtained by deprotonation [47]).

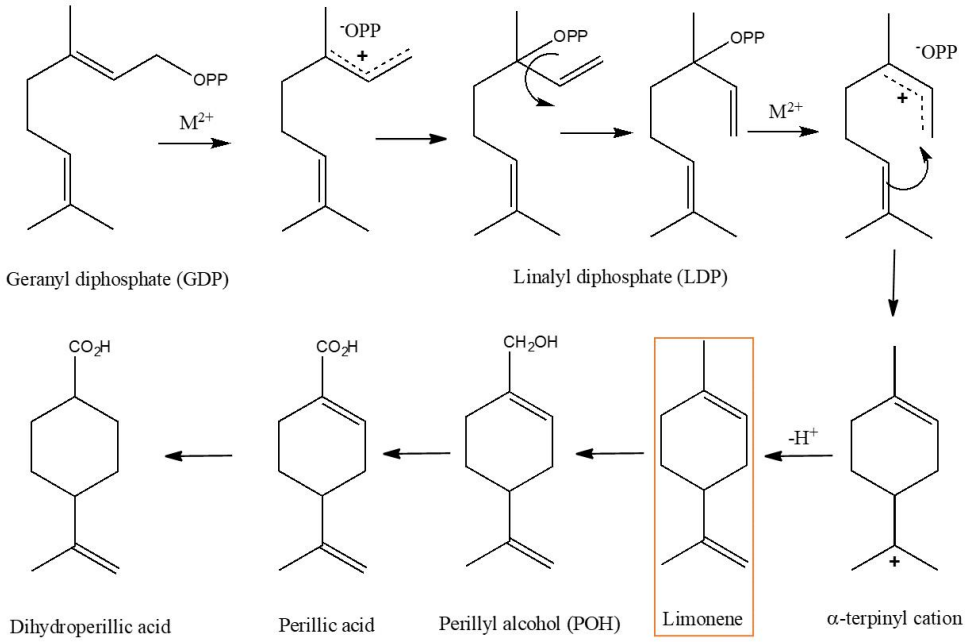

Figure 2. Generation of *d*-Limonene and of its perillyl derivatives (source: Mann J et al. in Natural products, their chemistry and biological significance. Longman Scientific & Technical 1994). *d*-Limonene and its metabolites have been shown to possess chemotherapeutic and chemo-preventive efficacy in various preclinical models of cancer.

8.1. Bio-availability & pharmacokinetics

d-Limonene is rapidly absorbed and metabolized [48,49]. One hour following oral absorption of radiolabeled *d*-Limonene (1g/kg), only 15% of circulating radioactivity is retrieved, 30% corresponding to the dihydroperillic acid and 50 % to perillic acid. This major metabolite being more active than *d*-Limonene itself, it may participate in the effects observed for *d*-Limonene. Oral absorption of the labeled product corresponds to distribution in most tissues followed by a urinary excretion, without accumulation effects. *d*-Limonene is also submitted to entero-hepatic re-absorption. The oral bio-availability of *d*-Limonene has been estimated at 43.0%. It has also been shown that an oral administration of *d*-Limonene at 200 mg/kg corresponds to a maximal plasma concentration of 11,3 µg/mL, which is obtained after 58 min. A biphasic evolution corresponding to the decrease of this plasma concentration as a function of time is characterized by a plasmatic half-life of 34 then 337 min [49].

8.2. Phase I studies and allergenicity

Pamela Crowell et al. [11] identified plasma metabolites of *d*-Limonene in the blood of seven healthy human volunteers having ingested 100 mg/kg *d*-Limonene without any adverse event nor side effect. Capillary gas chromatography/mass spectrometry analysis (GC/MS) indicated that at least five compounds were present in their plasma 4h after ingestion. Two major peaks were identified as the *d*-Limonene metabolites dihydroperillic acid and perillic acid, and two minor peaks

were found to be the respective methyl esters of these acids [11]. *d*- and *l*-Limonene are weak inhibitors of isoprenylation enzymes, while their major metabolites, perillic acid and POH, are potent inhibitors of the geranylgeranyl transferase type I enzyme and at a lesser level of farnesyl transferase [50]. Indeed *d*-Limonene has a pronounced chemotherapeutic activity and displayed minimal toxicity in preclinical studies. A phase I clinical trial was designed to establish its toxicity, the maximum tolerated dose (MTD) and pharmacokinetics in patients with advanced cancer. This phase I trial was followed by a limited phase II evaluation in breast cancer. *d*-Limonene was well tolerated in these patients at doses which may have clinical activity [51,52].

Besides its medical properties, the monoterpene *d*-Limonene is widely used in perfumes, soaps, and foods because of its pleasant fragrance. It is also extensively exploited in the cleaning industry for its strong solvent properties. *d*-Limonene is listed in the Code of Federal Regulation as GRAS (Generally Recognized As Safe) and precise concentrations are indicated for its usage as a flavoring agent. *d*-Limonene has been classified at level 3 by the International Cancer Research Center (CIRC), as "not classifiable as to its carcinogenicity to humans" [53]. In Europe, limonene is authorized in cosmetic products, but has been identified by the SCCS (Scientific Committee on Consumer Safety) as likely to cause allergic reactions in certain circumstances. It was consequently included on a list of 26 allergenic substances [54]. In fact, an extremely low rate of sensitization to 2% *d*-Limonene has been reported in a large study performed on 2396 subjects [55]. Only three subjects showed low or mild reaction yielding a rate of 0.1% risk of allergic reaction. The regulation (EC) nÆ1223/2009 on cosmetic products requires manufacturers to indicate the presence of certain allergenic substances in the list of ingredients if they are present above certain levels, in order to ensure that consumers are adequately informed. The presence of limonene must be mentioned in the list of ingredients on the labeling when its concentration exceeds 0.001% in "leave on the skin" products and 0.01% in products that are rinsed off the skin.

In fact, pure non-oxidized *d*-Limonene is completely inert and remarkably well tolerated. Only oxidized *d*-Limonene may become allergenic and irritating [56,57]. The extensive use of *d*-Limonene contained in cleaning, industrial or household solutions, as well as soaps, cosmetics, drinks, foods and drugs, as mentioned above, has prompted thorough investigations on how they affect the skin due to the observation of occupational allergy to oxidized *d*-Limonene in dermatitis patients [57–59]. However, the concentrations and conditions used by Bråred Christensson [57] of prolonged contact with artificially and extensively oxidized limonene indeed are not "real life". Moreover, even using these extreme conditions, only 5% of the subjects tested showed a reaction, noting that these were "consecutive dermatitis patients", not normal subjects. A EU regulation has been issued, allowing up to 20 mMol/L of the oxidized form of *d*-Limonene in such products, an amount only seldom reached.

9. Toxicity studies in animals and humans

d-Limonene has been shown to cause renal toxicity only in male rats in a series including various species and genders. No toxicity whatsoever was observed in female rats nor in mice, guinea pigs, rabbits and dogs of both genders. Of note *d*- and *l*-Limonene enantiomers are metabolized by P450 enzymes in liver microsomes. Studies with recombinant P450 enzymes suggest that there are species-related differences in the metabolism of limonene by P450 enzymes, particularly in the pathway from *d*-carveol to *d*-carvone. CYP2C enzymes were initially suggested to play major roles in metabolizing *d*-carveol to *d*-carvone and *d*-carvone to *d*-carveol by liver microsomes, since the

activities were inhibited significantly by anti-human CYP2C9 antibodies in animal species. Indeed, the male-specific rat CYP2C11 (but not female-specific CYP2C12) is able to convert limonene to carveol and perillyl alcohol [60]. Limonene enantiomers are converted to respective carveols, perillyl alcohols (POHs), and carvones (oxidative metabolites of carveols) by liver microsomes of dogs, rabbits, and guinea pigs. Mice, rats, monkeys, and humans produce carveols and POHs, but not carvones. Humans, monkeys, rats, and mice do not convert *d*-carveol to *d*-carvone, but are able to metabolize *d*-carvone to *d*-carveol, with male rats having the highest rates [60].

A specific toxicity has been reported in cats, with three publications alerting to acute episodes following *inhalation* of the active principle [61–63]. The second study was undertaken to determine the effects of a single *dermal* application of a commercial insecticidal dip containing the excessively high dose of 78.2% *d*-Limonene [62]. At the manufacturer's recommended concentration of 1.5 oz/gal of water, no clinical signs nor toxic lesions were observed. At 5 times the recommended concentration, clinical signs were mild and consisted of short-lived hyper-salivation, ataxia and muscle tremors resembling shivering. At 15 times the recommended concentration, clinical signs included hyper-salivation lasting for 15 to 30 minutes, moderate-to-severe ataxia lasting 1 to 5 hours, muscle tremors resembling shivering lasting 1 to 4 hours, and severe hypothermia beginning soon after treatment and lasting 5 hours. No deaths nor lasting effects were seen at any dosage.

10. Perspectives

Data reported above strongly suggest that such compounds as *d*-Limonene have a place among available solutions to counteract chronic inflammation in ailments explored in the models tested.

Yet, in the field of chronic diseases, two new elements have now to be taken into account. First, the discovery of the prominent role of the microbiota on global health has stimulated the commercialization of large amounts of new food supplements supporting digestive functions, such as probiotics. However, many other compounds, including *d*-Limonene, are liable to positively interact with the complex intestinal functions, as dietary supplements. Second, the recognition that many chronic diseases depend on the gut/microbiota imbalance has created a new transversality in the medical approach. Here again, this balance can be positively affected/restored by non toxic anti-inflammatory compounds such as *d*-Limonene.

In a different field, also explored as shown above, plant extracts, and especially *d*-Limonene, by interfering with the cells' structure, can improve skin elasticity and favor wound healing in a non-inflammatory context. An interaction with the skin microbiota is also likely to be involved in this context.

It must me noted, however, that market-access regulations have redefined the new field of nutritional supplementation, broadening it to mere pharmacologic treatment. The fact that nutraceutical products may have the same efficacy as drugs, including some side effects, has resulted in the issue of new and even more stringent laws that must be taken into consideration. That such regulations were deemed necessary yet underlines the fact that evidence-based data have been recognized, opening the field of alternative therapeutic solutions.

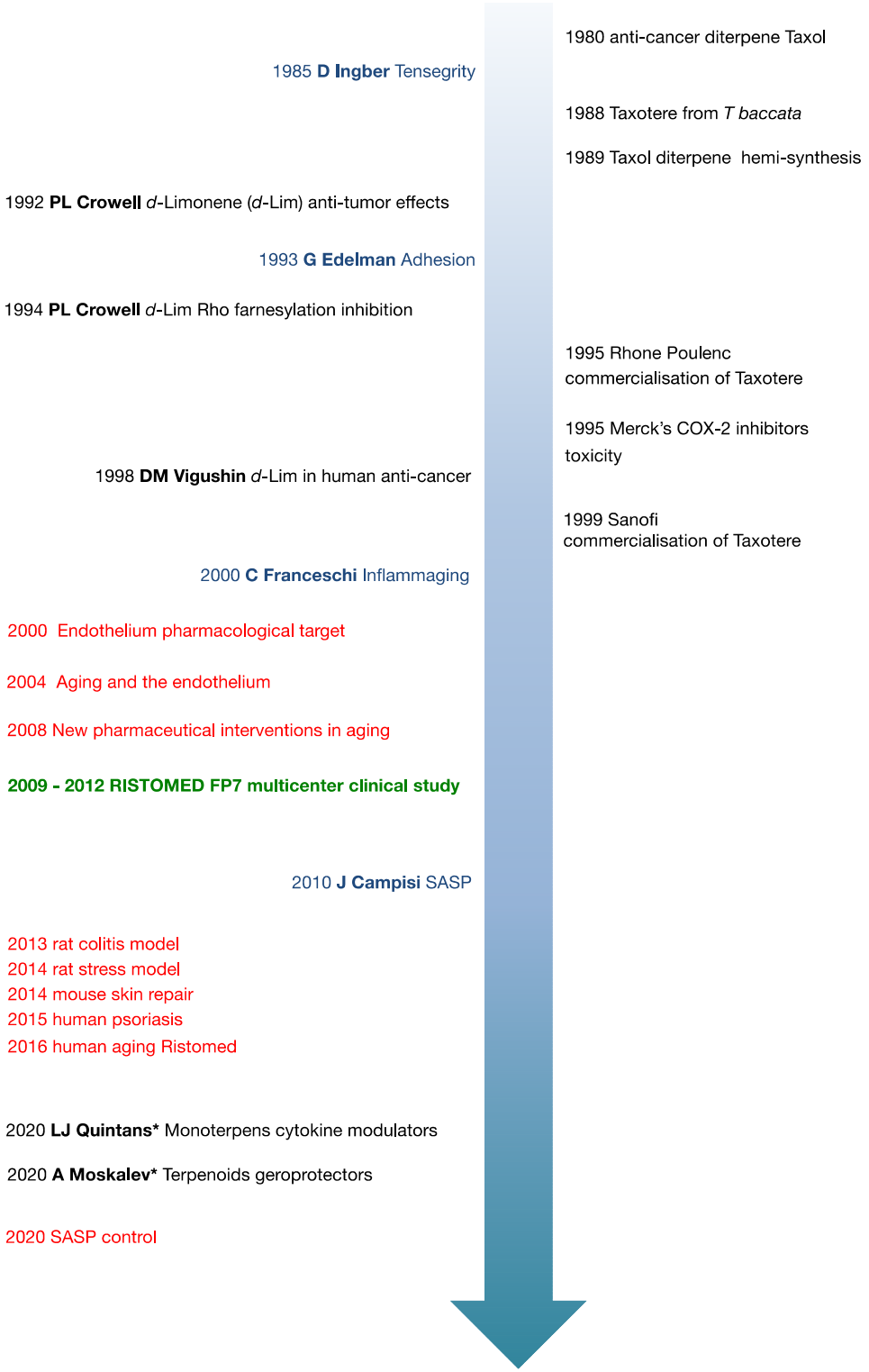

Figure 3. Timeline of key time points of terpenoid-based anti-cancer drug-discovery (top) and *d*-Limonene-related (bottom) research in inflammation and cell senescence. Key concepts are in black, inspiring authors in blue, research by corresponding author of this review in red with the European project RISTOMED in green. Asterisks indicate external reviews.

11. Further considerations on the place of monoterpenes in the anti-inflammatory arsenal

Monoterpenes, and in particular *d*-Limonene, have shown to be able to decrease levels of circulating cytokines, one of the major issues not only of chronic disease and stress but also of the more recent Covid-19 syndemic. Although dealt with by the brain and its HPA-axis, the spontaneous secretion of anti-inflammatory glucocorticoids turns out in the long run to disconnect the gut barrier tightness. Gut barrier dysfunction has been shown to be mechanistically involved in the spread of inflammation from the local immune-mucosal compartment to the whole body [64]. As a result of a long adaptive process, at some point the whole body becomes inflamed, the brain not being excluded and developing a frank neuro-inflammation. The most frequently reported symptoms are joint disease, gut pain, various auto-immune dermatitis, sleeplessness and depression. We have shown that *d*-Limonene addresses gut barrier dysfunction as well as mood disorders, primarily thanks to its capacity to lower the cytokine dependent inflammatory status, but also to its clear repair activity, that we reckon is in the first place simply a consequence of lowering the inflammatory status.

What does the current market propose to treat inflammatory disorders with their companion clinical issues ranging from psoriasis to Common Chronic Diseases (CCD) encompassing cardio-metabolic syndrome? Biologics and glucocorticoids, as well as, more recently, fluvoxamine, all address separate aspects of the inflammation conundrum (stress-pro-inflammatory cytokines-gut barrier impeachment-mood disorder). Yet none of these really covers the shifting forms of inflammatory issues, reaching from initial discomfort, either psychological or physical (itchy skin, gut pain) to frank QoL alteration, such as in the Chronic Fatigue Syndrome, sleeplessness or incurable psoriasis, and of course immune incompetence in front of a rather harmless virus such as SARS-CoV-2. AISA derived OPE compounds could indeed provide an answer in these complex contexts.

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