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26. New Anti–Inflammatory Molecule (Aisa 5203-l) Promotes Adaptive Strategies in Cell Aging

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Abstract

In our study an animal model simulating chronic intestinal inflammation was interesting to test *in vivo* the efficacy of anti-inflammatory applications of AISA molecules. Other chronic inflammatory diseases such as chronic pulmonary obstruction would equally be of interest for a pre-clinical development. Moreover, the association of *d*-Limonene with another active principle thoroughly selected, represents an opportunity to be explored. Geraniol and geraniol acetate, "pro-drug" of geraniol, part of the AISA molecules portfolio, are also well tolerated. *In vivo* evaluation however still requires the determination of their pharmacokinetics.

Keywords: Inflammation biology, chronic inflammatory disease, protein C, Il-6, TNF and anti-TNF activity; anti-inflammatory compounds; vascular endothelium; endothelial senescence; stress; adhesion molecules; actin cytoskeleton; monoterpens; *d*-Limonene

Introduction

Aging is a pleiotropic multi-factorial process, initially determined by genetic and environmental conditions. We have chosen to concentrate our attention to the measurement of the changes of endothelial cells in the course of the inflammatory process. In fact, the high concentration of pro-inflammatory cytokines will promote premature senescence of vascular lining of endothelial cells. Chronic inflammatory disease contributes to the deterioration of endothelial cell function further increasing their premature senescence. We have characterized a family of molecules able to inhibit inflammatory markers in endothelial cells and to reverse the consequences of replicative senescence. We have named these molecules "AISA" (Anti Inflammatory Senescence Actives). One of them ("AISA 5203-L") also showed a compelling anti-stress activity, as measured by a FOB (Functional Observation Battery), whereby analgesic effects were associated to enhanced motility and less irritability in non-pathological stress situations.

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Moreover, an exceptional capacity to restore both the epithelia of colon and dermal layer from pro-inflammatory agonists and toxic substances were described and patented. Most probably the protective effect is due to the capacity of AISA 5203-L to inhibit circulating Tumor Necrosis Factor-a (TNF-a), as well as Interlukin-6 (IL-6) and Interlukin-1 (IL-1), that are cytokines, most relevant to the inflammatory reaction that have been also put in relationship to the aging process.

Chronic Inflammation as Promoter of Age-Related Disease

Chronic inflammation is an underlying cause of many apparently unrelated, age-related diseases. As humans grow older, systemic inflammation can inflict devastating degenerative effects throughout the body. This fact is often overlooked, yet persuasive scientific evidence exists that correcting a chronic inflammatory disorder will enable many of the infirmities of aging to be prevented or reversed.

When we envisage a link between aging and recurrent or chronic inflammation, we refer to pathological consequences of inflammation in well-documented medical literature. Regrettably, the consequences of systemic inflammation continue to be ignored. By following specific prevention protocols, the inflammatory stimulation could be significantly reduced. An important role in preventing the onset of a chronic inflammatory condition has been attributed either to the practice of a physical activity or to the prescription of a personalized diet, or both. In the frame of the EU Capacities study RISTOMED (www.ristomed.eu), AISA Therapeutics is participating with the dietary supplementation of AISA 5203-L in addition to a controlled diet to a cohort of the elderly otherwise healthy persons (65-85 years).

Inflammatory Status and Auto-Immune and Degenerative Diseases

The immune function is affected in aging. Concomitantly, aging status results in an increase of inflammatory cytokines, such as TNF-a or Il-6. Inflammation-promoting cytokines are vital for tissue defense from microorganisms, but have been shown also to contribute to the progression of many degenerative diseases. Rheumatoid arthritis is a classic autoimmune disorder in which excess levels of cytokines such as IL-6, IL-1b and/or IL-8 are known to cause or contribute to the inflammatory syndrome (1-6).

But chronic inflammation is also involved in diseases associated to the metabolic syndrome resulting in atherosclerosis, heart valve dysfunction, obesity, diabetes, congestive heart failure, and gastro-intestinal diseases. Cancer and Alzheimer's diseases have both been shown to benefit from a systemic inflammation for their progression. In aged people with multiple degenerative diseases, the inflammatory marker C-reactive protein is often elevated, indicating the presence of an underlying inflammatory condition. Moreover, when a cytokine blood profile is conducted on people in a weakened condition, an excess level of one or more of the inflammatory cytokines, e.g., TNF-a, IL-6, IL-1b, as well as IL-8, are usually found.

Relevant Markers of Aging

In 2000 the New England Journal of Medicine published several studies showing that the blood indicators of inflammation are strong predictive factors for determining susceptibility to undergo a heart attack. Many international studies have validated this first communication (7-14).

A critical inflammatory marker is C-reactive protein. This marker indicates an increased risk for destabilized atherosclerotic plaque and abnormal blood clotting, which can lead to an acute heart attack. One of these studies (7) showed that people with high levels of C-reactive protein were almost three times as likely to die from a heart attack. This also implicates that elevated C-reactive protein, IL-6 and other inflammatory cytokines indicate significantly greater risks of contracting or dying from specific diseases (heart attack, stroke, Alzheimer's disease).

Moreover, C-reactive protein and IL-6 could also predict the risk of all-cause mortality as addressed by a study conducted on a sample of 1,293 healthy elderly people (8) followed for a period of 4.6 years. Higher IL-6 levels were associated with a twofold greater risk of death. Higher C-reactive protein was also associated with a greater risk of death, but to a lesser extent than elevated IL-6. Subjects with both high C-reactive protein and IL-6 were 2.6 times more likely to die during follow up than those with low levels of both of these measurements of inflammation. Concluding, it would seem C-reactive protein and IL-6 may be useful for identification of high-risk subgroups for anti-inflammatory interventions. Indeed, in 2003, the American Heart Association and Centers for Disease Control & Prevention (CDC) jointly endorsed the C-reactive protein test to screen for coronary-artery inflammation to identify patients at risk for heart attack.

Frailty in Elderly is Associated to Enhanced Inflammatory Profile

These results were further addressed by a new study on almost 5,000 elderly people (9) that was comparing frail seniors to their more active counterparts for the presence of increased inflammation markers. Associated to the elevated blood inflammatory markers, these frail seniors also tended to show an enhanced clotting activity, muscle weakness, fatigue and disability when compared to the active elderly people. We are not able to document, in how far these clinical outcomes are the origin or the consequence of inflammatory status, but once we recognize that they are interdependent, we can address them by prevention and treatment.

Collectively, these studies should motivate public health policies as well as conscious individuals to monitor their inflammatory status. If C-reactive protein is elevated, then the Inflammatory Cytokine Test Panel would also be highly recommended. Secondly, all those who suffer from any type of chronic disease may also consider to access to the Inflammatory Cytokine Test Panel in order to identify the specific inflammatory mediator that is causing or contributing to their health problem. In the aforementioned RISTOMED study, all the known pro-inflammatory markers are measured to characterize the specific degree of the inflammatory reaction in the population studied, as well as the capacity by AISA compounds to lower them. In fact at this moment a clinical trial conducted in University Hospitals of Berlin, Bordeaux and Rom is administrating low doses of AISA 5203-L daily to a cohort of 65-85 old people, and reports on its anti-inflammatory activity will be available in 2010.

Strategically Addressing Inflammatory Markers

For a long time the identification and production of anti-inflammatory drugs has concentrated on symptom remission, ignoring vascular mechanisms of inflammation and their consequences in the long term, such as increased cells' replicative senescence, and promoting degenerative disease. Now we know that the presence of pro-inflammatory markers in blood may in part be responsible for

degenerative disease, characteristic of aging. Through their clinical relevance, inflammatory markers can witness, via endothelial dysfunction, their implication in the occurring of disease (15-20).

In the past, monoterpenes, sesquiterpenes and diterpenes had been characterized by several authors as potential anti-cancer drugs on the basis of *in vitro* and *in vivo* studies, but their role as anti-inflammatory drugs has remained elusive.

In 2002, following our bio-guided selection, performed by means of our *in vitro* cell biology screening platform, we were able to identify four molecules out of 2000 as able to reverse inflammatory markers in senescent endothelial cells. Focusing on functional criteria, we were aiming at the identification of non-toxic molecules able to inhibit *in vitro*, the hallmarks of the inflammatory response, such as the expression of adhesive molecules (ICAM-1, VCAM-1, selectins), as well as the concomitant actin polymerization in endothelial cells. The four monoterpenes were contained in plants extracts (kindly provided by the University of Hanoi to the ICSN of the CNRS) originating from regional medicinal plants. The first patent has been filed in order to protect the therapeutic applications of "AISA" in pathologies associated with acute or chronic inflammatory stress. Two more international patents were filed protecting terpenes and in particular *d*-Limonene's clinical relevance in treating mood alterations and tissue repair. Moreover, our pre-clinical work brought up evidence for the consistency between *in vitro* and *in vivo* results concerning the anti-inflammatory effects of (4R)-1-methyl-4-isopropenylcyclohex-1-ene (*d*-Limonene), which we named "AISA 5203-L".

d-Limonene Pre-Clinical Studies

After an acute toxicity study and several dose-response studies aiming at the appreciation of the therapeutic window, pre-clincal studies were performed on a female rat TNBS induced colitis model and a murine SHK TPA model. These studies showed that the inhibition of adhesion molecules were comparable in the *in vitro/in vivo* experiments (21-23).

They also allowed us to establish that the therapeutic windows were corresponding to the *in vivo* pharmacologically active dose of 10 mg/kg given either *per os* or applied topically. Moreover, our *in vivo* data showed that plasma concentration of TNF-a is greatly reduced by the administration of *d*-Limonene and the score of post-lesional tissue regeneration enhanced and was comparable to that of ibuprofen**.** Unlike ibuprofen, AISA 5203-L also importantly contributes to pain tolerance and mood matching. Finally, on the quite differentiated capacity to elicit adhesion molecule expression follows TNF stimulation by different steroid and non steroid anti-inflammatory drugs (7-14).

Pharmacological Pertinence of Monoterpenes

Monoterpenes (31-37) are a class of isoprenoid molecules derived from the anabolism of acetate by the mevalonic acid branch biosynthetic pathways of plants. *d*-Limonene, a major component of orange peel oil, is formed by the cyclization of the 10-carbon isoprene intermediate geranylpyrophosphate. Interest in *d*-Limonene came from the ability of the compound to inhibit carcinogenesis in the murine benzo(*a*)pyrene-induced skin tumor model and inhibition of dibenzopyrene-induced s.c. sarcomas. The mechanisms by which *d*-Limonene and other cyclic monoterpenes inhibit tumor growth have not been firmly established. Geranylpyrophosphate, the isoprene intermediate from which these compounds are derived, is required for the synthesis of cholesterol, coenzyme Q (ubiquinone), and substrates used in the isoprenylation of several cellular proteins. Crowell *et al.,* (32) found that *d*-Limonene and other

monoterpenes inhibited isoprenylation of M_r 21,000–26,000 proteins, including $p21^{ras}$ and other members of the ras family of GTP-binding proteins that are involved in signal transduction and growth regulation. The post-translational isoprenylation of these and other proteins is an essential covalent modification that is required for protein localization and function. For example, farnesylation is required for plasma membrane association and signaling function of $p21^{ras}$. Other intracellular proteins require isoprenylation by addition of a farnesyl (15-carbon) or geranylgeranyl (20-carbon) group to the COOH terminus for localization to a cellular compartment or for interaction with other proteins. The four molecules, identified by the AISA Therapeutics cell biology platform, for their specific anti-inflammatory activity, following an *in vitro* screening on endothelial targets associating cyto – protective and adhesion inhibiting activities, turned out to be monoterpens: geraniol, geranyl acetate, d-limonene and iso-menthone, are intimately linked by a metabolic loop.

Although data available emphasized the anti-cancer activities of geraniol and *d*-Limonene, we were tempted to find out about the *in vitro/in vivo* consistency of our data in models adapted to the study of acute and chronic inflammation. In confirmation to our *in vitro* results, the capacity of geraniol (AISA 5202-G) to inhibit the adhesion of leukocytes following TNF-a stimulation had already been established. As for *d*-Limonene (AISA 5203-L), in consideration of the efficacy of its metabolite, perillyl alcohol (POH), already tested in clinical trials in patients with refractory solid malignancies, it seems plausible that it plays the role of a precursor. In conclusion, the complex sequence of events of the inflammatory response that includes endothelial adhesive molecule expression for the vascular recruitment of leukocytes to the site of injury, it seems to be concomitant with actin polymerization and challenge of the signaling pathway of the rho GTPase family. The activation of these proteins requires a post-translational iso-prenylation. *We think that the same mechanisms of action of the anti-cancer effects reported for geraniol and d-Limonene could equally be at the origin of their anti-inflammatory properties, reported here***.**

d-Limonene's Action on Stress

Important effects on mood in presence of stress situations had been documented by us previously in a rodent model thus motivating our choice to explore more in detail this unexpected effect (24-30).

As established by our Functional Observation Battery (FOB), *d*-Limonene was able to substantially contribute to pain tolerance and mood stabilization. The most intriguing result however that retained our attention, was the fact that the stressed animal (by a so-called non-pathological stress stimulating anxiety, comparable to maternal deprivation), instead of developing a freezing attitude, after oral administration of *d*-Limonene developed a "ludic" activity, starting to play with the wheel next to it. For us this was particularly interesting when compared to other mood or anxiety treating molecules, displaying substantially a hypnotic effect.

Discussion

To recapitulate links between inflammation, senescence and stress have been addressed in a fragmentary way whereas they should be considered by an integrated approach. Therefore identifying molecules able to prove anti- inflammatory/effective on replicative senescence and having a subtle but tangent effect on stress became a way to us to put this link in evidence. In this regard, I would evoke the historical and almost anecdotal properties of such molecules in food and recipes throughout the ages.

Linking Inflammation and Cell Senescence

Inflammatory diseases are numerous and systemic inflammation is a silent companion of stress and age. On the other hand, psychological stress in response to pain appears as an important customer of inflammation. The pharmacological strategies trying to inhibit inflammatory symptoms and related clinical episodes have been going far, and in case a proper indication is respected for their prescription, can be told to be successful, despite the recent side effects of several of them reported. But disease is, independently from its etio-pathology, a stressing agent by itself, able to promote inflammatory procrastination by not yet totally unraveled mechanisms. Unfortunately, a sustained anti-inflammatory treatment is inevitably associated to adverse effects, thus opening the field of the research and development of new less or not toxic and more tolerated ant-inflammatory strategies. In particular, we could provide evidence that the expression of vascular adhesion molecules is challenged by most used anti-inflammatory steroid and non-steroid drugs when compared to the effect of a triterpen contained in an edible plant used by Chinese populations since centuries to prevent rheumatoid arthritis (10).

Linking Inflammation and Stress

Recently, much attention has been given to stress as promoter of disease and syndromes implied in health decline and we have addressed this issue in a review and research article (3, 11).

Indeed, compounds found in natural environment, mostly plants, have acquired a new status of valid pharmacological candidates for the development of new drugs preventing, maintaining and curing on the basis of body integrity and substantially addressing lifestyle more than health. We think that if the aging process depends on genetic stability, metabolic control, and resistance to stress, longevity in particular seems related to this latter. If responses to stress anticipate adaptation to an unacceptable disparity between real or imagined personal experience and expectation, they include adaptive stress, anxiety, and depression. However, if stress persists, it may lead to chronic diseases, ranging from inflammation and cancer to degenerative diseases. If in the past 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. Uncontrolled inflammation is a critical defense mechanism, which in chronic conditions results in inflammatory pathogenesis, and in stressful conditions induces a diffuse (systemic) pro-inflammatory status. If sub-clinical chronic inflammation is an important pathogenic factor in the development of metabolic syndrome, then a cluster of common pathologies, including cardiovascular disease, will include markers associated with endothelial activation and dysfunction. Literature concerning monoterpenes essentially concentrate on the anti-cancer activities of *d*-limonene and geraniol, apparently due to the inhibiting activity of the isoprenoid biosynthesis and/or the inhibition of the transferases catalyzing the iso-prenylation of proteins. Our laboratory as well as others have well documented the capacity to modulate not only metastasis recruitment but also leukocyte recruitment via an inhibitory activity towards adhesive molecule expression, and thus the inflammatory reaction.

Possible Mode of Action of d-Limonene in Inflammation

d-limonene has been used by us in an *in vitro* model challenging human primary endothelial cells with the pro-inflammatory cytokine TNF-a and hydrogen peroxide (H_2O_2) for its capacity to down-regulate

endothelial adhesive molecules. In fact, in the course of the inflammatory response, peroxides, TNF-α, ICAM-1 expression and cytoskeleton proteins are all well-integrated elements of a complex sequence of events. In particular, ICAM-1 and cytoskeleton actin fibers interact via a signaling pathway implying the proteins of the rho GTPase family (35, 36). For their activation, these proteins require a post-translational isoprenylation, already described for *d*-limonene anti-cancer effects. This effect displayed by monoterpenes, could also be responsible for the resulting anti-inflammatory activity shown by our *in vitro* and *in vivo* studies. In fact adhesive molecules are expressed by endothelial cells, responding to a pro-inflammatory environment, which is always present in cancer at least at its dissemination stade. ICAM-1, VCAM-1 and P- and E-selectins are indeed able to recruit circulating metastasis, but are primarily devoted to the regulation of leukocyte diapedesis during immune survey and inflammatory reaction.

Possible Mode of Action of d-Limonene in Stress

The interesting results obtained on mood and pain modulation by *d*-Limonene supplementation, may rely on an effect displayed at the cytoskeleton level, though not at vascular endothelial but at neuronal level. Recently, the relevance of the significant contribution of actin cytoskeletal regulation to basal synaptic transmission and to various forms of synaptic plasticity has shown the great importance for functional responses (37). They imply molecular signaling machineries, able to coordinate real-time modifications in synapse activity and actin-dependent dynamics in pre- and post-synaptic terminals. Possibly, *d*-Limonene, which passes the BBB easily because of its reduced MW, contributes to the re-initialization of the functional and morphological plasticity of individual synapses (possibly within the limbic area), thus influencing neighboring synapses to affect the overall neuronal circuit. In fact in our view the quenching of endogenous opioid neuro-transmitters is blocked during inflammation because of stress fiber formation at the synaptic level, that would be de-polymerized by the monoterpenes action, thus permitting their liberation into the circulation, associated with a mood modulating effect.

Addressing Inflammation and Stress in Chorus

d-limonene has a tolerated pharmacokinetic profile, is rapidly metabolized into perillic and di-hydro-perillic acid or perillyl alcohol, monoterpenes themselves are more active than *d*-limonene itself, as reported by studies showing an interest of phase 1 pharmaco-kinetics of perillyl acid. With a biodisponibility of 43%, a plasmatic concentration of 11.3 mg/ml, after 58 min. and a bi-phasic T/2 corresponding to 34 and then 337 min., *d*-limonene would thus play the role of a pro-drug. In consideration of the relationship between mood alterations and inflammation, as seen in stress contributing to the development of a systemic inflammation as well as hypertension and atherosclerosis, we think that the mood stabilizing properties of AISA 5203-L permits us to extrapolate this drug as a candidate for anti-inflammatory applications.

Perspectives

It would be strategically interesting to confirm the anti-inflammatory mechanism of action of AISA molecules, by their impact on isoprenylation of proteins, measuring surrogate markers of the type ras farnesylation, as shown in anti-cancer studies (32). This type of study should also integrate the metabolites of AISA molecules, that could be more efficacious, as this has been shown for R-(+)-

Limonene and by our pre-clinical results on PA. Coming towards the end, the reader should be alerted about a few things that can be confirmed (38-42).

Today our study contributes to enhance the evidence for the relevance of a specific class of molecules contained in substances which may have been used either in the domesticated fruit and vegetable environment as food, such as the oleocantal ibuprofen – like molecule contained in olive oil (39), or as ritual substances, such as the incense and myrrh (42) containing anti-inflammatory and mood modulating terpenes. We presume that for centuries these raw materials were integrated in lean and sacred recipes devoted to the maintenance of health and the prevention of aging, because of their content in biologically active molecules, displaying their curing properties, either as anti-inflammatory remedies or inducing mood modulation allowing an enhanced perception of life.

Identifying a molecular target, this approach would result in a rational concept of original molecules ready to be patented as such, permitting to optimize their pharmacological efficacy. This vision would also contribute to better support the rational of the drug discovery project and to position the molecules as candidates in other therapeutic applications.

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