Title: THERAPEUTIC USE OF ALL-TRANS RETINOIC ACID (ATRA) IN PATIENTS SUFFERING FROM ALCOHOLIC LIVER DISEASE

Abstract: The current invention concerns a method for treating human patients suffering from Alcoholic Liver Disease (ALD) by administration of a retinoic acid receptor agonist (RAR) to said patients. In a preferred embodiment, the said RAR agonist is all-trans retinoic acid (ATRA). The current invention furthermore provides for a pharmaceutical composition, whereby said pharmaceutical composition can be used as a therapeutic to lower the levels of TNF-a in patients suffering from ALD and use of such composition.
THERAPEUTIC USE OF ALL-TRANS RETINOIC ACID (ATRA) IN PATIENTS SUFFERING FROM ALCOHOLIC LIVER DISEASE

TECHNICAL FIELD

The invention pertains to the technical field of therapeutics for patients suffering from alcoholic liver disease.

BACKGROUND

Alcoholic liver diseases (ALD) represent a major health problem. In Europe, 25% of the population has excessive alcohol consumption and 30% of them will develop hepatic complications, such as acute alcoholic hepatitis (AAH) which is associated with 20% of mortality despite the best current treatment (corticosteroids), and cirrhosis which represents the 8th cause of death in USA and for which the transplantation is the only curative option.

The spectrum of alcohol-related liver injury varies from simple steatosis to cirrhosis. Steatosis or fatty liver develops in 90% of individuals who drink more than 60g/day. It is usually asymptomatic and reversible after 4-6 weeks of abstinence. However, drinking more than 40-80g/day for an average of 25 years will lead to fibrosis or cirrhosis in 40-60% of patients. A subset of patients with chronic ALD will develop mild or severe acute hepatitis (AH), worsening their short-term prognosis. It is characterized by advanced liver disease combined with acute decompensation.

The amount of alcohol ingested is one of the several risk factors that have been identified to influence the development and progression of ALD. Type of alcohol and pattern of drinking may influence the risk of developing liver disease as well. Female gender, African-American and Hispanic ethnicities and genetic factors are all associated with ALD. Importantly, malnutrition has also an important role in determining the outcome of patients with ALD. Indeed, mortality is directly correlated to the degree of malnutrition and micronutrient abnormalities may aggravate the liver disease.

The currently accepted mechanism for ALD is based on a deregulation of cytokine metabolism. Several groups have demonstrated that monocytes from patients with AAH or alcoholic cirrhosis (AC) produce constitutively more TNF-α than cells from healthy subjects (HS), and even more after lipopolysaccharide (LPS) stimulation, etc. These increased levels of TNF-α are correlated to the development of systemic complications, the progression of the disease as well as to the cirrhosis-related
severity and mortality. Concordant with these human studies, complementary experiments in vitro and in vivo, on animal experimental models, helped to confirm the pivotal role played by TNF-a in the pathophysiology of ALD (reviewed in McClain Semin Liver Dis. 1999). The major stimuli for cytokine production are thought to be products of oxidative stress derived from alcohol metabolism and gut-derived bacterial components. Chronic alcohol consumption leads to increased gut permeability, helping bacterial translocation to the portal system. Bacterial components, as LPS, activate Kupffer cells, hepatic resident macrophages, through the Toll like Receptors, as TLR4, stimulating the production of inflammatory cytokines and particularly TNF-a.

The therapeutic options for ALD have been developed in regard to the pathological mechanisms involved but are based on the stage of the disease. In case of decompensation features, as encephalopathy, ascite, variceal bleeding, pulmonary, renal or infectious complications, treatments are mostly palliative and symptomatic, and extend the scope of this review. Unfortunately, focusing on specific treatments for ALD, we will realize that there are poor options and an urgent need to develop new therapeutic strategies.

Abstinence is the most important therapeutic intervention for patients with ALD, as it improves the outcome of hepatic injury, decreases progression to cirrhosis and improves survival at all stages of ALD.

In regard to the deleterious deregulated cytokine environment associated with AH, several agents have been studied, targeting specific cytokines, and particularly TNF-a. Despite promising results in an experimental animal model, the clinical trial evaluating the use of anti-TNF-a (Infliximab) in human AH has to be prematurely withdrawn because of increased infectious complications.

Pentoxifylline, a phosphodiesterase inhibitor, has been shown to inhibit biosynthesis of TNF-a, and as such have been studied as a treatment in AH and particularly when associated with hepato-renal syndrome. At present, the results are controversial and its use in severe AH is still debated. Glucocorticoid agonists are also known to block the synthesis of TNF-a, depressing the translation of its mRNA. Even if the precise mechanism of action of steroids in AH has not been completely elucidated, their anti-inflammatory role seems to be effective in increasing the short-term survival of patients with severe AH, characterized by a Maddrey discriminant function (MDF) >32, and represent the only treatment available for this severe and acute stage of the disease.
On the other hand, many therapeutic interventions targeting the oxidative stress have also been studied. But vitamin E, silymarin or combination of anti-oxidants have not been able to show a convincing benefit in AH. Although several strategies have been tested in the long-term management of ALD, none have proven efficacy. S-adenosyl-L-methionine, a precursor to glutathione, has been studied for its anti-oxidant potential, but the meta-analysis of nine randomized-controlled trials didn’t show any benefit in terms of mortality and complications. The same conclusion was reached with anti-inflammatory and anti-fibrotic Colchicine. It has to be noted that nutritional therapy is recommended at all stages of ALD. Indeed, proteino-energetic deficiency is a common feature of patients with ALD and the degree of malnutrition correlates with disease severity and outcomes in patients with AH as well as with the development of major complications in patients with stable cirrhosis (infection, encephalopathy and ascite). Deficiencies in micronutrients such as zinc, magnesium and Vitamin A are also frequent in ALD, but up to now, the studies have focused mainly on proteins and calories intakes.

All-trans retinoic acid (ATRA) is one of the active metabolites of Vitamin A (VA). It has long been known for its effects on embryonic development, cellular growth, and differentiation, but recently, its anti-inflammatory properties have gained more interest. ATRA and associated retinoids are lipophilic molecules that can pass through plasma membranes and enter the nucleus where they bind retinoic acid receptors (RARs). These receptors are members of the nuclear receptor family and can be divided into 2 subgroups, Retinoid Acid Receptor (RAR) and Retinoid X Receptor (RXR). ATRA can bind both but has higher affinity to RAR. Ligation of ATRA to its receptors induces allosteric changes that allow RARs to bind specific DNA recognition sites and regulate gene transcription. Anti-inflammatory mechanisms by which ATRA downregulates T helper 1 (Th1) cytokines, such as IFN-γ, TNF-a and IL-12, appear to involve inhibition of both AP-1- and NF-KB-dependent transcription as well as destabilization of TNF-a mRNA. ATRA is currently used in the treatment of several types of cancers, including acute promyelocytic leukemia, squamous cell carcinoma of the head and neck, and skin cancer, for its actions on cellular proliferation and differentiation. Besides these latter, ATRA has the capacity to repair emphysematous lung damage by generating new septa and increasing alveolar surface area in adult lung. As such, it has been evaluated in the treatment of human emphysema.

VA deficiency in alcoholic patients has been documented (Leo and Lieber, 1983). The mechanisms responsible for this depletion involve increased metabolism and
mobilization of VA from the liver, the competition of alcohol dehydrogenase (ADH)-catalyzed retinol oxidation, and the inhibition of retinoic acid biosynthesis.

The publication of Motomura et al. (2001) disclose that ATRA can suppress TNF-a expression by rat Kupffer cells in an experimental model of ALD, and by stimulated cultured rat hepatic macrophages (HM). The authors showed that 500 nM of ATRA caused a 50% inhibition in LPS stimulated TNF-a expression in normal rat HM, most probably through destabilization of TNF-a mRNA (Motomura et al., 2001). Moreover, they showed that on cells isolated from a rat model of alcoholic liver injury, ATRA could normalize the levels of TNF-a. The authors postulate in the article that ATRA is a potential candidate for treatment of liver disease where the presence of TNF-a and other pro-inflammatory cytokines play an important role. Pan et al. (2006) discloses that a low dose of ATRA can ameliorate ethanol-induced liver injuries in a rat model.

There remains a need in the prior art for an effective treatment without severe side effects for patients suffering from ALD. The present invention aims to provide for a therapeutic method for improving the health condition and symptoms in human patients suffering from ALD, as well as to diminish the episodes of decompensation. The present invention aims to lower the elevated levels of pro-inflammatory cytokines such as TNF-a usually linked to ALD without the use of a direct inhibitor of TNF-a. As known from previous studies with for instance the anti-TNF-a Infliximab, a direct action on the levels of TNF-a might cause severe and unwanted side-effects. Therefore, it is advisable to elucidate the mechanism behind the elevated levels of TNF-a in ALD patients. Although the prior art indicate that ATRA administration is possible treatment for patients suffering from ALD by lowering TNF-a, based on experimental data obtained in animal models, the latter could not yet been shown in human patients. Moreover, the extrapolation of these data to the human patient has shown to be rather difficult as although these experimental animal models tend to approach the human physiopathology as close as possible, the liver pathology that animals develop is not completely analogous to the clinical situation encountered in humans. The complexity of ALD, the implication of environmental, genetic and other risk factors, the progressive natural history of the disease, are difficult to implement in such models (Arteel GE. Dig. Dis., 2010). This has been thoroughly proved in prior art, by the Infliximab-case as mentioned above, whereby results obtained in a rat model of ALD, could not be readily extrapolated to human patients due to unforeseen and
unwanted side effects and non-effectiveness of the treatment (Iumuro et al., Hepatology, 1997; Naveau et al., Hepatology, 2004). It is the subject of the current invention to at least solve part of the problems as mentioned here above.

SUMMARY OF THE INVENTION

The present invention provides a method for treating human patients suffering from Alcoholic Liver Disease according to claim 1. In a preferred embodiment, said method involves the administering of a Retinoic Acid Receptor (RAR) agonist. Administering of said RAR agonist to human patients suffering from ALD is said to reduce the severity of the symptoms of ALD as well as reduce the periods of decompensation. In a preferred embodiment, the said RAR agonist is all-trans retinoic acid or ATRA.

In a further aspect, the invention provides for a pharmaceutical composition, whereby said the pharmaceutical composition can be used as a treatment for patients suffering from ALD and for lowering the levels of TNF-a in patients suffering from ALD according to claim 11. In a preferred embodiment, the said pharmaceutical comprises a RAR agonist. In a more preferred embodiment, the said pharmaceutical composition comprises ATRA.

The therapeutical activity of RAR agonists such as ATRA in human patients suffering from ALD were previously unknown. The ease of administration and the absence of side effects when administered to patients makes it a very attractive medicament for treating a growing amount of patients suffering from ALD or ALD related diseases.

DESCRIPTION OF FIGURES

Figure 1 depicts the plasmatic concentrations of retinol or vitamin A (Fig. 1A) and all-trans retinoic acid (ATRA) (Fig. 1B) in 10 healthy subjects (HS) and 10 alcoholic cirrhotic patients (AC). *p<0.01, ** p<0.001, HS versus AC.

Figure 2 depicts the TNF-a production in supernatants of peripheral blood mononuclear cells (PBMCs) from 10 HS and 10 AC, 24h after Con A or LPS stimulation (NS: non stimulated) (Fig. 2A) and TNF-a mRNA expression by PBMCs from 2 HS and 2 AC, after a 4h stimulation (Fig. 2B). * p<0.01, ** p<0.001, HS versus AC.
Figure 3 shows the TNF-a mRNA stability in LPS- (Fig. 3A) and Con A- (Fig. 3B) stimulated PBMCs from 7 Alcoholic Cirrhosis (AC; ⋅) and 4 Healthy Subjects (HS; A).

Figure 4 depicts the TNF-a production in supernatants of ALD patients PBMCs stimulated with LPS (Fig. 4A) or with Con A (Fig. 4B). Before stimulation, PBMCs were pre-treated for 1 h with ATRA or SR 11237 (RXR-agonist) or TTNPB (RAR-agonist). DMSO (1/10^6) was used as vehicle. * p<0.01 LPS versus LPS + ATRA/+ TTNPB; ** p<0.005 Con A vs Con A + ATRA.

Figure 5 depicts Alanine aminotransferase assessment in sera of mice. Ctrl: Control diet; OH: Ethanol diet; + Con A: + IV injection of Con A; + ATRA: + oral gavage of ATRA 3 times a week. Olive oil was used as the vehicle for ATRA. Ctrl (n=8); OH (n=12); Ctrl + Con A (n=12); OH + Con A (n=16); OH + Con A + ATRA (n=18) *p<0.05; ** p<0.001 (Pool of 2 independent experiments).

Figure 6 shows the assessment of LPS-induced activation of murine peritoneal macrophages isolated from vitamin A sufficient mice (VAS; □) and vitamin A deficient mice (VAD; ●). Fig. 6A depicts the TNF-a concentration and Fig. 6B the NO2 production in supernatants of cell culture in a pool of 3 independent experiments.

Figure 7 depicts a flow cytometry analysis of murine peritoneal macrophages. CD86 and CD80 expression on the surface of peritoneal macrophages isolated from vitamin A sufficient (VAS), vitamin A deficient (VAD) mice and VAD mice supplemented orally with ATRA was assessed. The graphs are representative of 2 independent experiments. Numbers in quadrants represent the percentage of cells among all peritoneal cells.

Figure 8 shows the activation of murine peritoneal macrophages in ATRA supplemented mice. LPS-induced activation of murine peritoneal macrophages isolated from vitamin A sufficient mice (VAS+oil;a), vitamin A deficient (VAD+oil; ⋅) mice receiving olive oil as vehicle control and VAD mice supplemented with ATRA (VAD+ATRA; ●) was assessed. FIG 8(A) shows TNF-a concentration and Fig 8(B) N02 production in supernatants of cell culture. (ND, not detectable)

DETAILED DESCRIPTION OF THE INVENTION

The present invention concerns for a method according to claim 1 to treat patients suffering from alcoholic liver disease (ALD).
In a second aspect, the present invention furthermore provides for a pharmaceutical composition according to claim 11.

Unless otherwise defined, all terms used in disclosing the invention, including technical and scientific terms, have the meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. By means of further guidance, term definitions are included to better appreciate the teaching of the present invention.

As used herein, the following terms have the following meanings:

The term "treatment", "treating" and the like, refer to obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment" as used herein, covers any treatment of a disease in a mammal, particularly in a human, and includes: (a) preventing the disease or a symptom of a disease from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it (e.g. including diseases that may be associated with or caused by a primary disease; (b) inhibiting the disease, i.e. arresting its development; and (c) relieving the disease, i.e. causing regression of the disease.

The term "inflammatory response" or "inflammation" refers to the type of response by the body to disease, the presence of pathogens and injury, a response characterized by the classical signs of experiencing pain, swelling, heat or localized warmth, loss of function and redness. Inflammation is a key part of the body's defence system and is accompanied by the production of pro-inflammatory cytokines.

The term "pro-inflammatory cytokines" refers to cytokines which are predominantly produced by activated immune cells and are involved in the amplification of inflammatory reactions.

The term "alcoholic liver disease (ALD)" relates to a heterogenous liver pathology which develops in general due to excessive alcohol abuse. Other factors that contribute to the development of ALD are genetic factors, personal susceptibility to ALD, sex, ethnic background and the toxicity of the consumed alcohol. ALD has three stages of liver damage: fatty liver (steatosis), alcoholic hepatitis (inflammation and
necrosis), and alcoholic liver cirrhosis. All are caused by chronic heavy alcohol ingestion.

The term "alcoholic steatosis" or "fatty liver" is defined as an excessive amount of fat in the hepatocytes due to excessive alcohol abuse.

The term "cirrhosis" relates to a histological diagnosis that involves replacement of the normal hepatic parenchyma with extensive thick bands of fibrous tissue and regenerative nodules. It results in the clinical manifestations of portal hypertension and liver insufficiency. Patients may present with jaundice, pruritus, abnormal laboratory findings (e.g., thrombocytopenia, hypoalbuminemia, coagulopathy, transaminases elevation ASAT > ALAT), or complications of portal hypertension, such as variceal bleeding, ascites, or hepatic encephalopathy. However, "compensated" alcoholic cirrhosis may be asymptomatic. Nor the symptoms and signs of alcoholic cirrhosis or the liver histological analysis are able to differentiate it from other causes of cirrhosis.

"Alcoholic cirrhosis" is defined in patients consuming more than 40 g/day of alcohol for men and more than 20 g/day for women with characteristics of cirrhosis and without other cause of liver disease (viral hepatitis (B, C or others virus), non-alcoholic steatohepatitis (obesity, metabolic syndrome, diabetes), auto-immune disease (auto-immune hepatitis, primitive biliary cirrhosis), hemochromatosis, Wilson disease). The severity of alcoholic cirrhosis is evaluated by 2 scores. The Child-Pugh score based on serum levels of bilirubin and albumin, on the prothrombin time, the presence of ascites and the degree of encephalopathy. The MELD score is based on the serum levels of creatinin and bilirubin, and on the prothrombin time.

"Alcoholic hepatitis" may be diagnosed concurrently or not with alcoholic cirrhosis. It is a histological diagnosis as well, characterised by Mallory bodies and hyalin necrosis, hepatocytes ballooning and inflammatory infiltrates, mostly neutrophils. It can be suspected on clinical and biological features as moderate fiever, abdominal pain, nausea, recent jaundice, moderate hepatic encephalopathy, hyperleucocytosis with neutrophils, elevation of transaminases, mostly ASAT, hyperbilirubinemia and decreased prothrombin time, in a context of excessive alcohol consumption. The Maddrey score, based on the prothrombin time and bilirubin, is used to evaluate the degree of severity. If the score is >32, the alcoholic hepatitis is considered severe and corticoids are started.
The term "decompensation" relates to a state of the liver whereby a threshold is reached with regards to repetitive liver damage and whereby the liver functions decline remarkably. Signs of liver decompensation include: abnormal clearance of proteins absorbed through the intestinal tract, leading to ammonia retention and hepatic encephalopathy; abnormal excretion, leading to an accumulation of bilirubin in the blood, producing jaundice; ascites: whereby increased sinusoidal pressure, as with severe inflammation or scarring of the liver, leads to fluid accumulation in the abdomen that becomes more difficult to control with progressive liver decompensation; portal hypertension whereby scarred liver tissue acts as a barrier to blood flow and causes increased portal blood pressure. A major consequence is the rupture of oesophageal varices, causing massive and potentially fatal bleeding.

The term "Child-Pugh score" refers to the test used to assess the prognosis of chronic liver disease, mainly cirrhosis, as well as the required strength of the treatment and the necessity of liver transplantation.

The term "INR" or "International normalized ratio" refers to a measure of the extrinsic pathway of coagulation. This measure is used to determine the degree of liver damage.

The term "MELD" or "Model for End-Stage Liver Disease" is a scoring system for assessing the severity of chronic liver disease and is used in the determination of prognosis and prioritizing for receipt of a liver transplant. The MELD score uses the patient's values for serum bilirubin, serum creatinin, and the INR to predict survival.

The following formula is used to calculate the MELD score:

\[
\text{MELD} = 3.78 \times \ln(\text{serum bilirubin (mg/dL)}) + 11.2 \times \ln(\text{INR}) + 9.57 \times \ln(\text{serum creatinin (mg/dL)}) + 6.43
\]

"A", "an", and "the" as used herein refers to both singular and plural referents unless the context clearly dictates otherwise. By way of example, "a compartment" refers to one or more than one compartment.

"About" as used herein referring to a measurable value such as a parameter, an amount, a temporal duration, and the like, is meant to encompass variations of +/- 20% or less, preferably +/-10% or less, more preferably +/-5% or less, even more preferably +/-1% or less, and still more preferably +/-0.1% or less of and from the specified value, in so far such variations are appropriate to perform in the disclosed
invention. However, it is to be understood that the value to which the modifier "about"
refers is itself also specifically disclosed.

"Comprise," "comprising," and "comprises" and "comprised of" as used herein are
synonymous with "include", "including", "includes" or "contain", "containing",
"contains" and are inclusive or open-ended terms that specifies the presence of what
follows e.g. component and do not exclude or preclude the presence of additional,
non-recited components, features, element, members, steps, known in the art or
disclosed therein.

The recitation of numerical ranges by endpoints includes all numbers and fractions
subsumed within that range, as well as the recited endpoints.

The expression "% by weight" (weight percent), here and throughout the description
unless otherwise defined, refers to the relative weight of the respective component
based on the overall weight of the formulation.

One aspect of the invention aims to decrease the development of complications in
patients suffering from ALD by administering a compound which helps restore the
normal immune response in these patients. The inventors conducted various
experiments to show that patients with ALD exhibit a deficiency in vitamin A (VA) and
its metabolite all-trans retinoic acid (ATRA). ATRA is an agonist of the retinoic acid
receptor (RAR) and retinoid X receptor (RXR).

Moreover, in a preferred embodiment of the current invention, the said upregulation of
TNF-α in ALD patients can be overcome by supplementation with a RAR agonist. In
more preferred embodiment, the said upregulation of TNF-α in ALD patients can be
overcome by supplementation of ATRA.

Administration of an RAR agonist to said human ALD patients reduces the
inflammatory response and will decrease the severity of alcoholic hepatitis.
Said the term "ATRA" as used herein is to be understood as all trans retinoic acid or salts of all trans retinoic acid, C1-C10 alkyl esters of all trans retinoic acid, salts of C1-C10 alkyl esters of all trans retinoic acid, C1-C10 alkyl amides of all trans retinoic acid, or salts of C1-C10 alkyl amides of all trans retinoic acid. ATRA is, amongst others, available as Atragen(R), Avita(R), Renova(R), Retin-A(R), Vesanoid(R), Vitinoin(R), Liposomal ATRA, Tretinoin Liposomal, AR-623, and Tretinoin(R).

An active agent (e.g., an RAR agonist, ATRA, or an additional therapeutic agent such as a corticosteroid, etc.) is administered to individuals in a formulation with a pharmaceutically acceptable excipient(s). The terms "active agent" and "therapeutic agent" are used interchangeably herein. A wide variety of pharmaceutically acceptable excipients is known in the art and need not be discussed in detail herein. Pharmaceutically acceptable excipients have been amply described in a variety of publications, including, for example, A. Gennaro (2000) "Remington: The Science and Practice of Pharmacy," 20th edition, Lippincott, Williams, & Wilkins; Pharmaceutical Dosage Forms and Drug Delivery Systems (1999) H. C. Ansel et al., eds., 7th ed., Lippincott, Williams, & Wilkins; and Handbook of Pharmaceutical Excipients (2000) A. H. Kibbe et al., eds., 3rd ed. Amer. Pharmaceutical Assoc.

The pharmaceutically acceptable excipients, such as vehicles, adjuvants, carriers or diluents, are readily available to the public. Moreover, pharmaceutically acceptable auxiliary substances, such as pH adjusting and buffering agents, tonicity adjusting agents, stabilizers, wetting agents and the like, are readily available to the public.

The said active agent may be administered to the host using any convenient means capable of resulting in the desired therapeutic effect. Thus, an active agent can be incorporated into a variety of formulations for therapeutic administration. More particularly, an active agent can be formulated into pharmaceutical compositions by combination with appropriate, pharmaceutically acceptable carriers or diluents, and may be formulated into preparations in solid, semi-solid, liquid or gaseous forms, such as tablets, capsules, powders, granules, ointments, solutions, suppositories, injections, inhalants and aerosols.

As such, administration of an active agent can be achieved in various ways, including oral, buccal, rectal, parenteral, intraperitoneal, intradermal, subcutaneous, intramuscular, transdermal, intratracheal, intravenous, etc., administration. In some embodiments, e.g., in a combination therapy as described herein, two different routes
of administration are used. For example, in some embodiments, a RAR agonist is administered intravenously, while a corticosteroid is administered orally.

Subcutaneous administration of a therapeutic agent can be accomplished using standard methods and devices, e.g., needle and syringe, a subcutaneous injection port delivery system, and the like. A combination of a subcutaneous injection port and a device for administration of a therapeutic agent to a patient through the port is referred to herein as "a subcutaneous injection port delivery system." In some embodiments, subcutaneous administration is achieved by a combination of devices, e.g., bolus delivery by needle and syringe, followed by delivery using a continuous delivery system.

In some embodiments, a therapeutic agent is delivered by a continuous delivery system. The terms "continuous delivery system," "controlled delivery system," and "controlled drug delivery device," are used interchangeably to refer to controlled drug delivery devices, and encompass pumps in combination with catheters, injection devices, and the like, a wide variety of which are known in the art.

Mechanical or electromechanical infusion pumps can also be suitable for use with the present invention. Examples of such devices include those described in, for example, U.S. Pat. Nos. 4,692,147; 4,360,019; 4,487,603; 4,360,019; 4,725,852; 5,820,589; 5,643,207; 6,198,966; and the like. In general, the present methods of drug delivery can be accomplished using any of a variety of refillable, pump systems. Pumps provide consistent, controlled release over time. Typically, the agent is in a liquid formulation in a drug-impermeable reservoir, and is delivered in a continuous fashion to the individual.

In one embodiment, the drug delivery system is an at least partially implantable device. The implantable device can be implanted at any suitable implantation site using methods and devices well known in the art. An implantation site is a site within the body of a subject at which a drug delivery device is introduced and positioned. Implantation sites include, but are not necessarily limited to a subdermal, subcutaneous, intramuscular, or other suitable site within a subject's body. Subcutaneous implantation sites are generally used because of convenience in implantation and removal of the drug delivery device.

Drug release devices suitable for use in the invention may be based on any of a variety of modes of operation. For example, the drug release device can be based
upon a diffusive system, a convective system, or an erodible system (e.g., an erosion-based system). For example, the drug release device can be an electrochemical pump, osmotic pump, an electro-osmotic pump, a vapor pressure pump, or osmotic bursting matrix, e.g., where the drug is incorporated into a polymer and the polymer provides for release of drug formulation concomitant with degradation of a drug-impregnated polymeric material (e.g., a biodegradable, drug-impregnated polymeric material). In other embodiments, the drug release device is based upon an electrodiffusion system, an electrolytic pump, an effervescent pump, a piezoelectric pump, a hydrolytic system, etc.

Drug release devices based upon a mechanical or electromechanical infusion pump are also suitable for use with the present invention. Examples of such devices include those described in, for example, U.S. Pat. Nos. 4,692,147; 4,360,019; 4,487,603; 4,360,019; 4,725,852, and the like. In general, a subject treatment method can be carried out using any of a variety of refillable, non-exchangeable pump systems. Pumps and other convective systems are in some embodiments used due to their generally more consistent, controlled release over time. Osmotic pumps are used in some embodiments due to their combined advantages of more consistent controlled release and relatively small size (see, e.g., PCT published application no. WO 97/27840 and U.S. Pat. Nos. 5,985,305 and 5,728,396)). Exemplary osmotically-driven devices suitable for use in a subject treatment method include, but are not necessarily limited to, those described in U.S. Pat. Nos. 3,760,984; 3,845,770; 3,916,899; 3,923,426; 3,987,790; 3,995,631; 3,916,899; 4,016,880; 4,036,228; 4,111,202; 4,111,203; 4,203,440; 4,203,442; 4,210,139; 4,327,725; 4,627,850; 4,865,845; 5,057,318; 5,059,423; 5,112,614; 5,137,727; 5,234,692; 5,234,693; 5,728,396; and the like.

In some embodiments, the drug delivery device is an implantable device. The drug delivery device can be implanted at any suitable implantation site using methods and devices well known in the art. As noted above, an implantation site is a site within the body of a subject at which a drug delivery device is introduced and positioned. Implantation sites include, but are not necessarily limited to a sub-dermal, subcutaneous, intramuscular, or other suitable site within a subject's body.

In some embodiments, a therapeutic agent is delivered using an implantable drug delivery system, e.g., a system that is programmable to provide for administration of a therapeutic agent. Exemplary programmable, implantable systems include
Implantable infusion pumps. Exemplary implantable infusion pumps, or devices useful in connection with such pumps, are described in, for example, U.S. Pat. Nos. 4,350,155; 5,443,450; 5,814,019; 5,976,109; 6,017,328; 6,171,276; 6,241,704; 6,464,687; 6,475,180; and 6,512,954. A further exemplary device that can be adapted for the present invention is the Synchromed infusion pump (Medtronic).

In pharmaceutical dosage forms, the agents may be administered in the form of their pharmaceutically acceptable salts, or they may also be used alone or in appropriate association, as well as in combination, with other pharmaceutically active compounds. The following methods and excipients are merely exemplary and are in no way limiting.

The agents can be formulated into preparations for injection by dissolving, suspending or emulsifying them in an aqueous or non-aqueous solvent, such as vegetable or other similar oils, synthetic aliphatic acid glycerides, esters of higher aliphatic acids or propylene glycol; and if desired, with conventional additives such as solubilizers, isotonic agents, suspending agents, emulsifying agents, stabilizers and preservatives.

For oral preparations, an active agent (e.g., an RAR agonist, an additional therapeutic agent such as an ACE inhibitor, a corticosteroid, etc.) is formulated alone or in combination with appropriate additives to make tablets, powders, granules or capsules, for example, with conventional additives, such as lactose, mannitol, corn starch or potato starch; with binders, such as crystalline cellulose, cellulose derivatives, acacia, corn starch or gelatin; with disintegrators, such as corn starch, potato starch or sodium carboxymethylcellulose; with lubricants, such as talc or magnesium stearate; and if desired, with diluents, buffering agents, moistening agents, preservatives, and flavoring agents.

Furthermore, an active agent can be made into suppositories by mixing with a variety of bases such as emulsifying bases or water-soluble bases. An active agent can be administered rectally via a suppository. The suppository can include vehicles such as cocoa butter, carbowaxes and polyethylene glycols, which melt at body temperature, yet are solidified at room temperature.

Unit dosage forms for oral or rectal administration such as syrups, elixirs, and suspensions may be provided wherein each dosage unit, for example, teaspoonful, tablespoonful, tablet or suppository, contains a predetermined amount of the composition containing one or more active agents. Similarly, unit dosage forms for
injection or intravenous administration may comprise the agent(s) in a composition as a solution in sterile water, normal saline or another pharmaceutically acceptable carrier.

The term "unit dosage form" or "dosage" as used herein, refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of an active agent (e.g., an RAR agonist, an additional therapeutic agent such as an ACE inhibitor, etc.) calculated in an amount sufficient to produce the desired effect in association with a pharmaceutically acceptable diluent, carrier or vehicle. The specifications for the unit dosage forms depend on the particular compound employed and the effect to be achieved, and the pharmacodynamics associated with each compound in the host.

In some embodiments, a subject method involves administering to an individual in need thereof an effective amount of a RAR agonist, as monotherapy.

In some embodiments, a RAR agonist is administered, where the RAR agonist is ATRA. In some embodiments, ATRA is administered in a dosage of from about 5 mg to about 100 mg per m² body surface per day, e.g., from about 5 mg/m² to about 10 mg/m², from about 10 mg/m² to about 15 mg/m², from about 15 mg/m² to about 20 mg/m², from about 20 mg/m² to about 25 mg/m², from about 25 mg/m² to about 30 mg/m², from about 30 mg/m² to about 35 mg/m², from about 35 mg/m² to about 40 mg/m², from about 40 mg/m² to about 45 mg/m², from about 45 mg/m² to about 50 mg/m², from about 40 mg/m² to about 60 mg/m², from about 60 mg/m² to about 70 mg/m², from about 70 mg/m² to about 80 mg/m², from about 80 mg/m² to about 90 mg/m², or from about 90 mg/m² to about 100 mg/m² per day. In many embodiments, the RAR agonist is administered orally.

In some embodiments, a RAR agonist, e.g. ATRA, is administered in a dosage of from about 1 mg/kg body weight to about 25 mg/kg body weight, e.g., from about 1 mg/kg to about 5 mg/kg, from about 2 mg/kg to about 5 mg/kg, from about 5 mg/kg to about 10 mg/kg, from about 10 mg/kg to about 15 mg/kg, from about 15 mg/kg to about 20 mg/kg, or from about 20 mg/kg to about 25 mg/kg daily, for a period of time from about 1 day to about 30 days, e.g., from about 1 day to about 7 days, from about 7 days to about 2 weeks, or from about 2 weeks to about 30 days. In other embodiments, ATRA is administered in a dosage of from about 2 mg/kg body weight to about 25 mg/kg body weight, e.g., from about 2 mg/kg to about 5 mg/kg, from about 5 mg/kg to about 10 mg/kg, from about 10 mg/kg to about 15 mg/kg, from
about 15 mg/kg to about 20 mg/kg, or from about 20 mg/kg to about 25 mg/kg daily, for a period of time from about 1 day to about 1 year, or longer, e.g., from about 1 day to about 7 days, from about 7 days to about 2 weeks, or from about 2 weeks to about 1 month, from about 1 month to about 2 months, from about 2 months to about 3 months, from about 3 months to about 6 months, or from about 6 months to about 12 months, or longer.

In many embodiments, the RAR agonist (e.g. ATRA), is administered for a period of from about 1 day to about 7 days, or about 1 week to about 2 weeks, or about 2 weeks to about 3 weeks, or about 3 weeks to about 4 weeks, or about 1 month to about 2 months, or about 3 months to about 4 months, or about 4 months to about 6 months, or about 6 months to about 8 months, or about 8 months to about 12 months, or at least one year, and may be administered over longer periods of time.

The RAR agonist (e.g. ATRA) is administered at various frequencies, e.g., once per month, twice per month, three times per month, every other week (qow), once per week (qw), twice per week (biw), three times per week (tiw), four times per week, five times per week, six times per week, every other day (qod), daily (qd), or twice per day, substantially continuously, or continuously, and over a period of time ranging from about one day to about one week, from about two weeks to about four weeks, from about one month to about two months, from about two months to about four months, from about four months to about six months, from about six months to about eight months, from about eight months to about 1 year, from about 1 year to about 2 years, or from about 2 years to about 4 years, or more.

In another aspect, the invention provides for a novel therapeutic or pharmaceutical composition, whereby said pharmaceutical composition can be used as treatment in patients suffering from ALD. Said ALD comprises steatosis, alcoholic hepatitis or alcoholic liver cirrhosis. The pharmaceutical composition is used to lower the levels of TNF-a in patients suffering from ALD. In a most preferred embodiment, said pharmaceutical comprises an RAR agonist. Said composition comprises at least one of the specific chemical substances and compounds selected from the group consisting of all trans retinoic acid, salts of all trans retinoic acid, C1-C10 alkyl esters of all trans retinoic acid, salts of C1-C10 alkyl esters of all trans retinoic acid, C1-C10 alkyl amides of all trans retinoic acid or salts of C1-C10 alkyl amides of all trans retinoic acid. In a most preferred embodiment, the said pharmaceutical composition comprises all trans retinoic acid.
According to still further aspect of the present invention, the novel therapeutic composition contains from 0.01 to 0.15 % by weight, particularly from 0.02 to 0.05 % by weight of the specific chemical substances and compounds or "active agent(s)". The above mentioned chemical substances and compounds may be combined with further compounds like an ACE inhibitor or corticoids.

Said pharmaceutical composition may further comprise pharmaceutically acceptable carriers, excipients and/or diluents.

In case the pharmaceutical composition is for oral application, according to a further embodiment of the present invention, the therapeutic agent (or agents) is (are) administered in the form of tablets or capsules. Such tablets or capsules may contain from 1 to 300 mg, preferably from 1 to 150 mg, more preferably from 1 to 100 mg, and particularly from 1 to 50 mg of the active agent or agents.

Another possible way of applying a therapeutically effective amount of at least one of the above-mentioned specific substances to an individual (patient) is by means of including the substance (s) into liposomes and administering the liposomes to the individual, for instance by intravenous administration. Liposomes are spherical particles having typically a diameter of about 25 nm to about 5 um. Liposomes usually comprise one or more concentric lipid double layers having an aqueous interior compartment (so-called "lipid vesicles"). Liposomes are known as carriers for pharmaceutical substances, which can be selectively enriched in certain organs and cellular tissues by means of the liposomes, see e.g. Adv. Drug Deliv. Rev. 19,425 to 444 (1996) and Science 267, 1275 et seq. (1995).

Preferably, an intravenous dose unit of the pharmaceutical composition comprises from 5 to 200 mg/ m² active agents, more preferably from 5 to 150 mg/ m² active agents, more preferably, 5 to 100 mg/ m², more preferably 20 to 80 mg/ m², more preferably from 25 to 50 mg/ m².

Routes of administration of pharmaceutical composition according to the current invention to an individual may include oral and parenteral, including dermal, intradermal, intragastral, intracutan, intravasal, intravenous, intramuscular, intraperitoneal, intranasal, intravaginal, intrabuccal, percutan, rectal, subcutaneous, sublingual, topical or transdermal application, but are not limited the these ways of administration. For instance, the preferred preparations are in administrable form
which is suitable for oral application. These administrable forms, for example, include pills, tablets, film tablets, coated tablets, capsules, powders and deposits. Administration to an individual may be in a single dose or in repeated administrations, and may be in any of a variety of physiologically acceptable salt forms, and/or with an acceptable pharmaceutical carrier, binder, lubricant, excipient, diluent and/or adjuvant. Pharmaceutically acceptable salt forms and standard pharmaceutical formulation techniques are well known to persons skilled in the art.

It is readily apparent to those skilled in the art that other suitable modifications and adaptations of the compositions and methods of the invention described herein are evident and may be made without departing from the scope of the invention or the embodiments disclosed herein. Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included for purposes of illustration only and are not intended to limit the invention.

The present invention will be now described in more details, referring to experimental data and examples that are not limitative.

EXPERIMENTAL DATA

The following data rose from the experiments performed by the inventors, whereby it is shown that ATRA levels are lowered in patients suffering from ALD and that the latter causes an upregulation of pro-inflammatory cytokines such as TNF-α. ATRA administration to these patients can decrease the severity of the disease and serve as treatment for ALD. Data are expressed as median [min-max]. The significance of the difference between the groups was assessed using Mann-Whitney U test. Calculations were performed with the SPSS 19.0 software (Chicago, IL).

15 consecutive patients admitted in our institution with biopsy-proven alcoholic liver disease were studied. Exclusion criteria were: liver co-morbidities, as viral hepatitis and components of non-alcoholic steatohepatitis (NASH), treatment by antibiotics or steroids. The clinical characteristics of these patients are shown in Table 1. Blood samples were taken by peripheral puncture or through the jugular vein during trans-jugular hepatic biopsy. Serum was store at -20°C, protected from light, until assay. Peripheral blood mononuclear cells (PBMCs) were freshly isolated.
The study was performed with the approval of the Erasme Hospital Ethics Committee. Written informed consent was obtained from each patients and healthy volunteers.

Table 1. Table of patients. (AAH: Acute Alcoholic Hepatitis; HVPG: Hepatic Venous Pressure Gradient)

<table>
<thead>
<tr>
<th>Patients (n=15)</th>
<th>Median (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>57 (46-63)</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>5/10</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>3.3 (2.4-3.7)</td>
</tr>
<tr>
<td>Total Bilirubin (mg/dl)</td>
<td>3.9 (0.98-6.7)</td>
</tr>
<tr>
<td>INR</td>
<td>1.75 (1.4-2.04)</td>
</tr>
<tr>
<td>Creatinin (mg/dl)</td>
<td>1 (0.7-1.5)</td>
</tr>
<tr>
<td>Child-Pugh score</td>
<td>9 (7-11)</td>
</tr>
<tr>
<td>MELD score</td>
<td>20 (13-23)</td>
</tr>
<tr>
<td>AAH</td>
<td>4</td>
</tr>
<tr>
<td>HVPG</td>
<td>18.5 (17-21.8)</td>
</tr>
</tbody>
</table>

VA and ATRA deficiency in patients with ALP

Figure 1 shows the plasmatic levels of VA and ATRA, which were assessed in 10 consecutive patients with biopsy-proven stable alcoholic liver cirrhosis and 10 healthy subjects (HS). Determination of plasmatic concentrations of retinol and ATRA were carried out by high-performance liquid chromatography (HPLC). As previously reported (Leo et al., 1983), patients with alcoholic liver disease (ALD) disclose vitamin A (VA) deficiency, but for the first time, the inventors show that ATRA levels are significantly decreased as well (Patients VA: 143 [46-519] ng/ml; ATRA: 1 [0.8-1.2] ng/ml vs HS VA: 1275 [1100-1870] ng/ml; ATRA: 1.2 [1-1.4] ng/ml; p<0.001 and p<0.01, respectively) (see Fig. 1).

Increased LPS or Con A-induced production of TNF-γ by PBMCs of ALD patients

Peripheral blood mononuclear cells (PBMCs) were isolated from the same patients from which we measured VA and ATRA concentrations. PBMCs were isolated by Ficoll-Paque PLUS (GE Healthcare, Sweden) density gradient centrifugation. The isolated cells were subsequently washed 3 times in Hank’s Buffered salt solution (HBSS) and resuspended in RPMI 1640 supplemented with 10% heat-inactivated FBS, 1% penicillin 10 000 U/ml and 1% streptomycin 10 000 U/ml (Lonza). Viability was assessed by the trypan blue exclusion test and always exceeded 95%.
To compare production of Tumor Necrosis Factor (TNF)-α by stimulated PBMCs from ALD patients and HS, 2.5 × 10^6 cells were stimulated with 1 μg/ml lipopolysaccharide (LPS; Escherichia coli serotype 055:B5, Sigma) or 2.5 μg/ml Concanavalin A (Con A; Sigma). After 4h of incubation, cells were harvested and stored in lysis buffer at -80°C until mRNA extraction. Supernatants were collected 24h after stimulation and stored at -20°C until assays.

To determine ATRA and its agonists effects on TNF-α production, the same protocol was used but before stimulation, the cells were pre-treated for 1 h with 100 nM ATRA (Sigma) or 100 nM TTNPB (RAR-pan-agonist; Sigma) or 100 nM SR 11237 (RXR-pan-agonist; Tocris), all dissolved in DMSO.

ELISAs used to quantify human and mouse TNF-α (Duoset, R&D systems) had a sensitivity of 15.8 pg/ml and 31.3 pg/ml respectively.

Total RNA was extracted from stored PBMCs using a commercially available kit (Magna Pure LC mRNA HS kit, Roche Diagnostics, Basel, Switzerland) following manufacturer’s instructions. One step RT-PCR for human TNF-α and Hypoxanthine-guanine Phosphoribosyltransferase (HPRT) were performed on LightCycler 480 (Roche Diagnostics) following designated protocol. Probe, sense and antisense PCR primer sequences were the following: TNF-α: 5' - TGGCCAGCCAGTCAGTACATC - 3', 5' - CCCAGGGACCTCTCTAATC - 3', 5' - ACTTTGCTTTCCTTGGTCAGG - 3'; HPRT: 5' - TTTCACCAGCAAGCTTGCGACCTTGA - 3', 5' - AGTTCTGGCTTATATCACTCG - 3' and 5' - GACTTTGCTTTCCTGGTCAGG - 3'.

The inventors confirmed that LPS-stimulated PBMCs from ALD patients produce significantly more TNF-α than healthy PBMCs (7358.2 [3246.3-9100] pg/ml vs 1945.8 [343.6-5975] pg/ml; p<0.001). For the first time in humans, the inventors showed the same results following Con A stimulation (6695.6 [1082-9100] pg/ml vs 2280.8 [93.3-3981.7] pg/ml; p<0.01) (Fig. 2A).

The increased production of TNF-α in ALD patients has been attributed mainly to monocytes/macrophages activated by bacterial components coming from the gut, such as LPS. However, a growing body of evidences let suppose that lymphocytes may play an important role in the pathophysiology of ALD, as well, and might be another source of pro-inflammatory cytokines. In the current experiments, 2 types of stimulation were used: LPS, which activates preferentially macrophages through TLR4 ligation, and Con A, a polyclonal mitogen, that stimulates T cells.

Regarding these results, figure 2 A and B shows that patients with ALD disclosing VA and ATRA deficiencies, exhibit an excessive inflammatory response to both ConA and LPS stimulations. In the same way that is has been described in rats, ethanol seems to
sensitize immune cells, macrophages as well as T lymphocytes, to show an exacerbated response.

Increased TNF-α mRNA stability in PBMCs from ALP patients

The effects of chronic alcohol on the stability of TNF-α mRNA was previously studied (Motomura et al., 2001; Kishore et al., 2001). It was shown that chronic ethanol exposure increases TNF-α mRNA half-life, in vitro, on murine macrophages cultured with ethanol, and in vivo, on Kupffer cells isolated from rodents submitted to an ethanol diet, and they proposed it as a mechanistic explanation for increased TNF-α in ALD.

Regarding these elements in animals, the inventors studied TNF-α mRNA stability in ALD patients. Total RNA was extracted from stored PBMCs using a commercially available kit (Magna Pure LC mRNA HS kit, Roche Diagnostics, Basel, Switzerland) following manufacturer's instructions. RNA stability was assessed as follows: PBMCs from ALD patients and HS were stimulated with LPS (1 µg/ml). After 4h, actinomycin D (5 µg/ml; Sigma) was added to the cells, and every 15 min for 90 min, cells were harvested and stored at -80°C in lysis buffer until mRNA extraction and RT-PCR. The expression of TNF-α mRNA was standardized by that of HPRT mRNA, and the mRNA decay was assessed by expressing the data as the percentage of the mRNA level at time 0 for each group. GraphPad was used to produce a one phase decay non-linear regression and to calculate the half-life.

Figure 3A shows that following LPS stimulation, TNF-α mRNA remains stable in PBMCs from cirrhotic patients compared to its decay observed in HS. In the same way, figure 3B shows that Con A stimulation of alcoholic cirrhotic PBMCs is associated with an increased half-life of TNF-α mRNA compared to HS (T2-life post-LPS stimulation : ALD : not measurable vs HS : 22.3 min ; T2-life post-Con A stimulation : ALD : 171.4 min vs HS : 13.6 min).

Decreased TNF-α production by ATRA or RAR-agonist pre-treated PBMCs from ALD patients

To study the possible link between increased TNF-α and ATRA deficiency, the inventors evaluated the effect of ATRA supplementation in vitro on the production of TNF-α by PBMCs isolated from alcoholic cirrhotic patients. ATRA pre-treatment significantly decreased LPS-induced TNF-α production (w/o ATRA: 7358.2 [3246.4-9100] pg/ml vs with ATRA: 3207.6 [1158.2-7735.4] pg/ml; p<0.01) as well as Con A-stimulated production (w/o ATRA: 6695.6 [1082-9100] pg/ml vs with ATRA: 1841.1 [81.3-4426.8] pg/ml; p<0.005).
To determine if the pathway involved was more likely RAR (Retinoic Acid Receptor)- or RXR (Retinoic X Receptor)-dependent, the same experiments were made using TTNPB as a RAR-pan-agonist or SR 11237 as a RXR-pan-agonist and used the same dose than ATRA, 100 nM. TTNPB decreased TNF-a stimulated production similarly as ATRA, but SR 11237 had no effect, after both stimulations (LPS stimulation: +TTNPB: 3672 [2119-5865] pg/ml; +SR 11237: 8108.6 [5360-16000] pg/ml; Con A stimulation: +TTNPB: 1553 [1320.6-7617.5] pg/ml; +SR 11237 4949 [2063.1-7040.5] pg/ml) (Fig. 4). These results indicate that ex vivo ATRA pre-treatment is able to diminish significantly the production of the pro-inflammatory cytokine, TNF-a. This effect seems to be RAR-dependent rather than RXR (see Fig. 4A and B).

Decreased severity of a murine model of alcohol-induced liver sensitization by oral administration of ATRA

The modified Lieber-DiCarli diet administered for 10 days to mice induces hepatomegaly, increased activity of serum transaminases and macrovacuolar steatosis and is used as an experimental model for human ALD. The Lieber-DeCarli diet induces alcohol-related liver injury in mice, mostly characterized by steatosis. Jaruga et al. (2004) have demonstrated that injection of Con A to alcohol-fed mice enhances hepatic inflammation and necrosis compared to control diet-fed mice. In the current invention, an adapted experimental model of the alcohol-sensitized Con A-induced hepatitis was used. Eight-week-old female C57Bl6/J mice (Charles River, Brussels, Belgium) were fed the modified Lieber-DeCarli ethanol diet for 10 days. On the last day, mice were intra-venously injected with 15 mg/kg Con A and killed 8h later. To determine the in vivo effect of ATRA, mice were orally administered with 0.5 mg/mice ATRA or olive oil as vehicle 3 times a week starting on the first day of alcohol diet. Mice were killed by cervical dislocation, blood was sampled and liver excised and weighed. Part of it was fixed in formaldehyde, embedded in paraffin and stained with hematoxylin-eosin; the remainder was frozen at -80°C. The severity of liver injury was assessed by measuring serum levels of alanine aminotransferase (ALT) with a commercially available kit (Roche/Hitachi) based on methods recommended by the International Federation of Clinical Chemistry (see figure 5). The inventors combined this model with the IV injection of Con A to induce a more inflammatory alcoholic-sensitized liver disease.

The assessment of serum alanine aminotransferase (ALT) showed that oral supplementation of ATRA decreased the severity of Con A-induced hepatitis on alcohol-sensitized liver (w/o ATRA: 1960 [116-9048] IU/L vs with ATRA: 306 [88-5160] IU/L; p=0.055) (Fig. 5).
In this model, ethanol-fed mice are supposed to present a more severe Con A-induced hepatitis than control-fed mice, as ethanol primes T lymphocytes to an exaggerated response to the stimulation. In alcoholic cirrhotic patients, immune cells seem primed, as well, to produce more pro-inflammatory cytokines following stimulation than do PBMCs from healthy controls. The in vivo results let to suppose that the oral supplementation of ATRA is able to temper the inflammatory response induced by Con A, as mice present a less severe hepatitis.

**Increased LPS-induced activation of macrophages from VAD mice**

To evaluate the hypothesis that VA deficiency is associated with the pro-inflammatory status disclosed by ALD patients, the inventors generated VA deficient mice (VAD) and studied LPS-induced activation of their macrophages. Peritoneal macrophages (PM) were isolated from 8-10 weeks old VAD and VAS mice following the protocol according to Riordan et al., 2003. Five milliliters of cold HBSS were injected twice intraperitoneal through a catheter and cell suspension was collected and centrifuged for 10 min at 400 x g. Supernatant was discarded and erythrocytes were lysed by adding 1 ml of ACK for 1.5 min. Thereafter, PM were immediately resuspended and centrifuged in HBSS. The resulting pellet was resuspended in culture medium consisting of RPMI 1640 supplemented with 10% FBS and 1% Penicillin/Streptomycin. Cells were seeded at 1.10^6 cells/ml and were further purified by removing non-adherent cells after a 2h incubation at 37°C in 5% CO2 atmosphere. For PM culture experiments, LPS diluted in culture medium was added to the wells at increasing concentrations, as indicated on the graphs. After a 24h stimulation, supernatants were harvested and stored at -20°C until TNF-a and NO analysis. NO production was evaluated by measuring nitrite, its stable degradation product, by the Griess' reaction. Interestingly, figures 6A and B show that macrophages from VAD mice produce more TNF-a and more NO2 than macrophages from VA sufficient mice following LPS stimulation. Again, this element shows a link between VA/ATRA deficiency and hyper-responsiveness of the immune system.

**Increased LPS-induced activation of macrophages from VAD mice**

Generating vitamin A deficient (VAD) mice, we have shown that their peritoneal macrophages (PerMO) produce more TNF-a and N02 after LPS stimulation than do PerMO isolated from vitamin A sufficient (VAS) mice, suggesting a role for vitamin A in the function of macrophages. Furthermore, we studied membrane expression of macrophage markers of activation, namely CD80 and CD86. By means of flow cytometry, we showed that the proportion of cells expressing CD11b+CD80+ and
CDllb+CD86+ (activated macrophages) was higher among cells isolated from the peritoneum of VAD mice than of VAS mice (CDllb+CD80+ VAS: 23.8 [12.9-34.7]% vs. VAD: 42 [38.7-45.3]% of PerMO; CDllb+CD86+ VAS: 32.6 [30.4-34.8]% vs. VAD: 52.4 [48.2-56.7]% of PerMO) (Figure 7). These observations are in favour of a qualitative as well as a quantitative effect of vitamin A on murine macrophages. Vitamin A deficiency not only increased the activation of macrophages, it also increased their number.

Of note, among VAD PerMO, we observed 2 distinct populations in terms of CD80 and CD86 intensity of expression as compared to the VAS PerMO where there was only one population (Figure 7). When we measured the mean fluorescence intensity (MFI) of CD80high and CD86high, it appeared that the MFI were higher in VAD PerMO than in VAS (CD80 MFI VAS: 3707.0 vs. VAD: 7119.4; CD86 MFI VAS: 3764.1 vs. VAD: 13079.9) (Figure 7), suggesting a role for vitamin A on the intensity of expression of surface markers of activation as well.

**Decreased activation and function of VAD macrophages after in vivo supplementation with ATRA**

Having demonstrated increased activation and proportion of VAD PerMO as compared to VAS PerMO, we wished to confirm the role played by the active metabolite of vitamin A, ATRA, in the immune response. We conducted in vivo experiments in which we gave ATRA to VAD mice or olive oil, as a vehicle control, to VAD and VAS mice. ATRA was given orally at the dose of 0.5 mg/mice diluted in 200 µl of sterile olive oil. Mice were supplemented every two days for 10 days. At the end of the experiment, mice were killed by cervical dislocation, PerMO were isolated and studied for their function and their activation. We observed that after ATRA supplementation, all variables studied in VAD mice tended to resemble to those of VAS mice. Indeed, LPS-induced production of TNF-a and N02 by PerMO was decreased after ATRA supplementation (Figure 8). In addition, the proportion of CDllb+CD80+ (34.3% of peritoneal cells) and CDllb+CD86+ (35.2% of peritoneal cells) decreased as well, and the two cellular populations observed in VAD PerMO (CDllb+CD80medium/high and CDllb+CD86medium/high) gave way to one population as in VAS PerMO and their MFI decreased (VAD+ATRA CD80 MFI: 3528; CD86 MFI: 3663.9) (Figure 7).

In humans, CD80 and CD86 are markedly increased in peripheral blood monocytes from cirrhotic patients as compared to healthy controls. Albillos et al. reported that alcoholic cirrhotic (AC) patients have increased number of circulating monocytes as compared to healthy subjects (HS) and that these cells are
more activated, expressing more CD80 and CD86 at their surface and producing more TNF-α. They assumed that gut bacterial translocation may partially explain the immune system cell abnormalities and TNF-α overproduction observed in AC patients as antibiotic therapy decreased, but do not normalize, the proportion of activated monocytes. Having demonstrated that vitamin A acts on activation and function of murine PerMO and that AC patients disclose vitamin A deficiency, we wondered if the latter could participate to immune abnormalities in humans as well. We isolated peripheral blood mononuclear cells (PBMCs) from 7 consecutive AC patients and 7 HS and analysed the expression of CD80 and CD86 (surface markers of activation) by CD14+ cells (monocytes). Flow cytometry analyses showed that the proportion of CD14+CD80+ and CD14+CD86+ cells was significantly higher among PBMCs from patients than from HS, at baseline and after LPS stimulation (Table 2). Interestingly, LPS stimulation induced a significant increase in the proportion of double positive cells CD14+CD80+ and CD14+CD86+ in controls (baseline vs. LPS stimulation: \( p=0.028 \) and \( p=0.046 \), respectively) and in patients (baseline vs. LPS stimulation: \( p=0.043 \) both) as well as of CD14+ in patients (\( p=0.043 \)). However, this increase did not reach significance for the proportion of CD14+ among PBMCs from controls (\( p=0.116 \)), suggesting the presence of an immune priming in AC patients capable of recruiting more monocytes after stimulation than HS. Intensity of expression of CD80 and CD86 tended to be higher on PBMCs from patients than from HS at baseline, but the difference did not reach significance, and was no more evident after 24 h of LPS stimulation (Table 2).
### Table 2

<table>
<thead>
<tr>
<th>At baseline</th>
<th>HS (n=4-7)</th>
<th>AC (n=4-7)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14⁺ CD80⁺ (%)</td>
<td>0.07 [0.01-0.12]</td>
<td>0.13 [0.12-0.59]</td>
<td>0.037*</td>
</tr>
<tr>
<td>CD14⁺ CD86⁺ (%)</td>
<td>10.8 [10.4-13.3]</td>
<td>20.6 [13.3-29.6]</td>
<td>0.021*</td>
</tr>
<tr>
<td>CD80 (MFI)</td>
<td>651 [531-1134]</td>
<td>1175 [637-2322]</td>
<td>0.076</td>
</tr>
<tr>
<td>CD86 (MFI)</td>
<td>2274 [1936-3156]</td>
<td>3302 [2299-5251]</td>
<td>0.076</td>
</tr>
<tr>
<td>After 24 h of LPS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD14⁺ CD80⁺ (%)</td>
<td>8.7 [5.9-14.5]</td>
<td>21.3 [16.7-23.4]</td>
<td>0.011*</td>
</tr>
<tr>
<td>CD14⁺ CD86⁺ (%)</td>
<td>18.4 [10.4-23.7]</td>
<td>30.8 [15.3-36.1]</td>
<td>0.028*</td>
</tr>
<tr>
<td>CD80 (MFI)</td>
<td>2192 [1307-3847]</td>
<td>2136 [1160-3848]</td>
<td>0.749</td>
</tr>
<tr>
<td>CD86 (MFI)</td>
<td>2719 [2135-4012]</td>
<td>2910 [1514-3599]</td>
<td>0.848</td>
</tr>
</tbody>
</table>

In conclusion, human data confirmed the increased activation of AC patients’ immune system. In regard to the murine results, we see that vitamin A deficiency play a role in these immune abnormalities and that ATRA supplementation can normalize them.

Administration of ATRA to cirrhotic patients reduces the inflammatory response.

11 AC patients were given a regime of 45mg/m² ATRA per day for 2 to 4 weeks by oral administration. A control group of 7 AC patients were given a placebo. Peripheral blood mononuclear cells (PBMCs) were isolated and analysed for the expression of CD80 and CD86 (surface markers of activation) by CD14⁺ cells (monocytes). Flow cytometry analyses showed that the proportion of CD14⁺CD80⁺ and CD14⁺CD86⁺ cells was significantly lowered among PBMCs from the patient group receiving ATRA compared to the same group before supplementation and to the control group.
Furthermore, TNF-alpha production was assessed in the supernatants of LPS-stimulated PBMCs isolated from patients having received ATRA, before and after supplementation. Results showed that oral ATRA supplementation in AC patients decreased TNF-alpha production as compared to baseline. This confirms that ATRA supplementation can reduce the inflammatory response in human patients suffering from ALD.

EXAMPLES

The following examples present therapeutic, pharmaceutical compositions according to the current invention. It is to be understood that these are merely to be seen as examples and are not limitative for the current invention.

Example 1

Fill mass for soft gelatin capsules:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATRA*</td>
<td>5-50 mg</td>
</tr>
<tr>
<td>Oil**</td>
<td>1-3 parts</td>
</tr>
<tr>
<td>Wax mixture***</td>
<td>1-5 parts</td>
</tr>
<tr>
<td>Fill volume</td>
<td>1-6 minis</td>
</tr>
</tbody>
</table>

*all trans retinoic acid or salts of all trans retinoic acid, CI-CIO alkyl esters of all trans retinoic acid, salts of CI-CIO alkyl esters of all trans retinoic acid, CI-CIO alkyl amides of all trans retinoic acid, or salts of CI-CIO alkyl amides of all trans retinoic acid

**natural vegetable oils, e.g. soy oil, peanut oil, and artificial glycerides

***composition of natural and artificial waxes or partially hydrated fats

Example 2

Hard gelatine capsules containing 20 mg active substance:

One capsule contains the following composition:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATRA*</td>
<td>20 mg</td>
</tr>
<tr>
<td>Gelatine Bloom</td>
<td>70 mg</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>108 mg</td>
</tr>
<tr>
<td>dl-alpha-Tocopherol</td>
<td>2 mg</td>
</tr>
<tr>
<td>Sodium ascorbate</td>
<td>10 mg</td>
</tr>
</tbody>
</table>
Microcrystalline cellulose 48 mg
Magnesium stearate 2 mg
(weight capsule content) 260 mg
*all trans retinoic acid or salts of all trans retinoic acid, Cl-CIO alkyl esters of all trans retinoic acid, salts of Cl-CIO alkyl esters of all trans retinoic acid, Cl-CIO alkyl amides of all trans retinoic acid, or salts of Cl-CIO alkyl amides of all trans retinoic acid

Procedure: The active substance is wet milled in a solution of gelatine, maltodextrin, dl-alpha-Tocopherol and sodium ascorbate. The wet milled suspension is spray-dried. The spray-dried powder is mixed with microcrystalline cellulose and magnesium stearate. 260 mg each of this mixture are filled into hard gelatine capsules of suitable size and colour.

Example 3

Tablet containing 20 mg active substance:

Composition:
Tablet kernel:
ATRA* 20mg
Anhydrous lactose 130.5 mg
Microcrystalline Cellulose 80.0 mg
dl-alpha-Tocopherol 2.0 mg
Sodium ascorbate 10.0 mg
Polyvinylpyrrolidone K30 5.0 mg
Magnesium stearate 2.5 mg
(Kernel weight) 250.0 mg
*all trans retinoic acid or salts of all trans retinoic acid, C1-C10 alkyl esters of all trans retinoic acid, salts of C1-C10 alkyl esters of all trans retinoic acid, C1-C10 alkyl amides of all trans retinoic acid, or salts of C1-C10 alkyl amides of all trans retinoic acid

Film coat:
Hydroxypropyl methylcellulose 3.5 mg
Polyethyleneglycol 6000 0.8 mg
Talc 1.3 mg
Iron oxide, yellow 0.8 mg
Titanium dioxide 0.8 mg
(weight of the film) 7.4 mg
Procedure: ATRA* is mixed with anhydrous lactose and micro-crystalline cellulose. The granular material is mixed with magnesium stearate and afterwards pressed as kernels with 250 mg weight. The kernels are film coated with a solution/suspension of above-mentioned film coat composition.

Example 4
Sachet containing 50 mg active substance

Composition:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATRA*</td>
<td>50.0 mg</td>
</tr>
<tr>
<td>Lactose, fine powder</td>
<td>990.0 mg</td>
</tr>
<tr>
<td>Microcrystalline Cellulose</td>
<td>1400.0 mg</td>
</tr>
<tr>
<td>Sodium Carboxymethyl-cellulose</td>
<td>14.0 mg</td>
</tr>
<tr>
<td>dl-alpha-Tocopherol</td>
<td>5.0 mg</td>
</tr>
<tr>
<td>Sodium ascorbate</td>
<td>20.0 mg</td>
</tr>
<tr>
<td>Polyvinylpyrrolidone K30</td>
<td>10.0 mg</td>
</tr>
<tr>
<td>Magnesium stearate</td>
<td>10.0 mg</td>
</tr>
<tr>
<td>Flavouring Agents</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>(Fill weight of a sachet)</td>
<td>2500.0 mg</td>
</tr>
</tbody>
</table>

*all trans retinoic acid or salts of all trans retinoic acid, C1-C10 alkyl esters of all trans retinoic acid, salts of C1-C10 alkyl esters of all trans retinoic acid, C1-C10 alkyl amides of all trans retinoic acid, or salts of C1-C10 alkyl amides of all trans retinoic acid

Procedure:

ATRA* is mixed with lactose, microcrystalline cellulose and sodium carboxymethyl cellulose. The mixture is granulated in water with a solution/dispersion of polyvinylpyrrolidone, dl-alpha-Tocopherol and sodium ascorbate. The granule is mixed with magnesium stearate and flavoring agents. It is filled into sachets of suitable size.
CLAIMS

1. A method for treating Alcoholic Liver Disease in human patients characterized in that a retinoic acid receptor agonist (RAR) is administered to said patients.

2. A method according to claim 1, characterized in that said patients suffer from steatosis, alcoholic hepatitis or alcoholic liver cirrhosis.

3. The method according to 1, characterized in that said RAR agonist is all trans retinoic acid (ATRA).

4. The method according to claim 1 characterized in that said RAR agonist reduces the produced levels of the pro-inflammatory cytokine TNF-a.

5. The method according to claim 1, characterized in that said RAR agonist is administered in a pharmaceutical composition whereby said composition is in a solid, semi-solid, liquid or gaseous form.

6. The method according to claim 1, whereby administration of said RAR agonist can be by orally, buccally, rectally, parenterally, intravenously, intraperitoneally, intradermal, subcutaneously, intramuscularly, trans-dermally, or intratracheally.

7. The method according to any of the claims 1 to 6, characterized in that said RAR agonist is orally administered to said patient.

8. The method according to claim 1, characterized in that said RAR agonist is intravenously administered to said patient.

9. The method according to claim 1, whereby said RAR agonist is administered in a dosage of from about 5 mg to about 100 mg per m² body surface per day.

10. The method according to claim 1, whereby said RAR agonist is administered in a dosage of from about 1 mg/kg body weight to about 25 mg/kg body weight.

11. A pharmaceutical composition, characterized in that said the pharmaceutical composition comprises a RAR agonist as active agent in a therapeutic amount for treating human patients suffering from ALD.

12. Pharmaceutical composition according to claim 11, characterized in that said patients suffer from steatosis, alcoholic hepatitis or alcoholic liver cirrhosis.

13. Pharmaceutical composition according to claim 11, characterized in that said RAR agonist is ATRA.

14. Pharmaceutical composition according to claim 11, characterized in that said composition comprises from 0.01 to 0.15% by weight of the active agent(s).

15. Pharmaceutical composition according to claim 11, characterized in that said composition comprises from 0.02 to 0.05% by weight of the active agent(s).

16. Pharmaceutical composition according to claim 11, characterized in that said composition is for oral application.
17. Pharmaceutical composition according to claim 11, characterized in that an oral dosage unit of said composition contains from 1 to 300 mg, more preferably 1 to 150 mg of said active agent(s).

18. Pharmaceutical composition according to claim 11, characterized in that said composition is for intravenous administration.

19. Pharmaceutical composition according to claim 11, characterized in that an intravenous dose unit of said composition comprises from 5 to 100 mg/m^2 of said active agent(s).

20. Pharmaceutical composition according to claim 11, characterized in that said active agent(s) are liposomal.

21. Use of a pharmaceutical composition according to claim 11 for reducing the inflammatory response in human patients suffering from Alcoholic Liver Disease.

22. A method for lowering TNF-a production in a patient suffering from ALD, characterized in that a retinoic acid receptor agonist (RAR) is administered to said patient.

23. A method according to claim 22, characterized in that said RAR agonist is all-trans retinoic acid (ATRA).
Figures

FIG. 1A

![Graph showing ROL (ng/ml) for HS (n=10) and AC (n=10).]

FIG. 1B

![Graph showing ATRA (ng/ml) for HS (n=10) and AC (n=10).]
FIG. 5

![Graph showing bar chart with different groups and p-values.]

- **: p=0.19
- *: p=0.055
- **: p=0.005
FIG. 7

VAS

VAD

VAD + ATRA

Comp-PE-A:: cd86

Comp-APC-A:: CD11b

Comp-FITC-A:: cd60

Comp-APC-A:: CD40
FIG. 8

![Graph showing TNF-α and NO2 levels with different LPS concentrations for VAS+oil, VAD+oil, and VAD+ATRA treatments.]

- **TNF-α (pg/ml)**
  - Axes: LPS (ng/ml) vs. TNF-α (pg/ml)
  - VAS+oil, VAD+oil, VAD+ATRA treatments

- **NO2 (µM)**
  - Axes: LPS (ng/ml) vs. NO2 (µM)
  - VAS+oil, VAD+oil, VAD+ATRA treatments
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/07 A61P1/16

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>PAN ZHIHONG; DAN ZI LI; FU YU; TANG WANGXIAN; LIN JUSHENG: &quot;Low-dose ATRA supl e mmentati on abol is h es P R M formati on in rat l i ver and amel i orates ethanol -i nduced l i ver i njury. &quot;, JOURNAL OF HUAZHONG UNIVERSITY OF SCI ENCE AND TECHNOLOGY, MEDICAL SCI ENCES, vol . 26, no. 5, 2006, pages 508-512, XP002682048, see &quot;Abstract and Discussion&quot;</td>
<td>1-23</td>
</tr>
<tr>
<td>A</td>
<td>CRABB ETAL: &quot;Alcohol and reti noi ds&quot;, ALCOHOLISM CLINICAL AND EXPERIMENTAL RESEARCH, vol . 25, no. 5 Suppl., 2001, pages 207s-217s, XP002682049, see &quot;Summary&quot;</td>
<td>1-23</td>
</tr>
</tbody>
</table>

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:
  *"A" document defining the general state of the art which is not considered to be of particular relevance
  *"E" earlier application or patent but published on or after the international filing date
  *"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  *"O" document referring to an oral disclosure, use, exhibition or other means
  *"P" document published prior to the international filing date but later than the priority date claimed
  *"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
  *"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
  *"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
  *"Z" document member of the same patent family

Date of the actual completion of the international search: 21 August 2012
Date of mailing of the international search report: 06/09/2012

Name and mailing address of the ISA/Authorized officer:
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016
Cattel 1, James

Form PCT/ISA210 (second sheet) (April 2005)