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(54) Title: METHOD OF TREATMENT OF LIVER DISEASE

(57) Abstract: A method of treatment of a subject suffering from liver disease comprising the administration of (i) an oral slow-release formulation of a calcium channel blocker with antioxidant effects and (ii) at least one flavonolignan.
Method of Treatment of Liver Disease

Field
The present invention relates to a method of treatment of liver disease and its complications including cirrhosis of the liver and portal hypertension. In particular, the invention relates to a method of treatment of non-malignant liver disease such as viral or toxic hepatitis, and fatty liver, where direct or indirect damage to the liver results, and to compositions for use in treatment of liver disease.

Background
Non-malignant liver diseases include toxic hepatitis such as alcoholic hepatitis, which is the most common form of toxicity, hepatitis caused by infection such as viral hepatitis, fatty liver, and less commonly, the immunological liver diseases characterised by chronic inflammation.

Therapeutic attempts to improve liver function have always been secondary to treatment of the primary disease processes, but there have been many attempts to improve function by targeting different components of the pathological processes which include cell swelling and hypoxia caused by oxidation of membrane cell membrane phospholipid and changes in cell membrane permeability. One key method employed has been the use of antioxidants beginning with the flavonolignans such as herbal extracts silymarin and silibinin (reviewed by Flora et al, 1998). However, the efficacy of such flavonolignans has been disappointing in alcoholic liver disease (Angulo et al, 2000), and there is the potential for hepatotoxic effects (Bass; 1999). The side effects of silymarin are most severe in diseased patients such as those with viral hepatitis C and patients with non-alcoholic fatty liver disease in which case the levels of accumulated flavanolignans have been reported as four or five times or more than that of healthy subjects (Schrieber et al., Drug Metab. Dispos. 2008 Sept. 36(9)1909-16). The side effects of silymarin include upset stomach, nausea, mild headaches, diarrhoea, vomiting and joint pain. Silymarin has also been reported
to reduce the effectiveness of oral contraceptives and in less common cases the side effects can include severe stomach pain, sweating and muscle weakness. More recently, tocopherol, dipyridamole (Novikov et al, 1991; Vargas et al, 2001), and a range of modern, both synthetic and naturally-occurring, antioxidants have been used (Vaidya et al. 1996).

Included among these are the calcium blocking agents verapamil, diltiazem, and amlodipine (Mason et al, 1999), and also nitrendipine (Thurman and colleagues, 1998). At first, it was thought that these agents acted directly on liver cells to block calcium entry as they do in excitable tissues such as the myocardium and in arteries (Liang and Thurman, 1992), however, it was soon realised that the liver does not have voltage-gated calcium channels, which are the target of these drugs in excitable tissues. Therefore, if these drugs were acting on the liver, they needed to act in a different way. Thus, it was found that many calcium blockers were also powerful antioxidants (Heo et al., 1997).

It has also been proposed that calcium channel blockers may dilate the hepatic artery to increase delivery of oxygenated blood to the liver (McLean. 1998). However, the protective effect of drugs such as verapamil, diltiazem (Liang and Thurman, 1992; Romero and colleagues, 1994) and the other calcium blockers occurs in isolated cells, and are therefore independent of drug-induced alteration in blood flow. The effects on hepatic arterial blood flow of calcium blockers administered orally in low doses have not been confirmed. Diltiazem has also been shown to have no effect on microvascular blood flow within the liver (Marteau and colleagues, 1988).

The discussion of the background to the invention herein is included to explain the context of the invention. This is not to be taken as an admission that any of the material referred to was published, known or part of the common general knowledge as at the priority date of any of the claims.
Summary

We have found that the co-administration of a calcium channel blocker (particularly diltiazem) that exerts its calcium blocking effect on mitochondria and has an antioxidant effect, and a flavonolignan which may be sourced from natural products, provide a significant improvement in the function of liver cells that have been treated to produce changes similar to those which occur in chronic liver disease.

It is believed that the effect of calcium channel blockers and in particular, diltiazem in diseased cells, enables cells to more adequately metabolise flavonolignans thereby reducing the potential for side effects.

Accordingly, we provide a method of treatment for patients suffering from liver disease comprising the administration of (i) an oral slow-release formulation of a calcium channel blocker with antioxidant effects and (ii) at least one flavonolignan.

In a preferred embodiment calcium channel blocker is diltiazem and is administered at a dose in the range of from 10 to 70 mg/day.

In another embodiment there is provided a method of treatment of a patient suffering from liver disease comprising the administration of (i) an oral slow-release formulation of a calcium channel blocker with antioxidant effects and (ii) an antioxidant effective amount of flavonolignan.

The flavonolignan is generally administered in an amount of from 1 to 5000mg per day.

In one embodiment thiamine is also administered and typical doses used may be in the range of from 1 to 200mg per day and preferably in the range of from 20 to 200mg thiamine per day.
The calcium channel blocker and flavonolignin may be present in the same unit dosage or may be administered separately. The diltiazem is in a slow release formulation which in one embodiment also includes the flavonolignin and in another embodiment does not include the flavonolignin which is administered separately either in a slow release or non slow release formulation.

The invention further provides a pharmaceutical composition for treatment or prevention of liver disease comprising (i) a calcium channel blocker with antioxidant effects and (ii) at least one flavonolignan. The pharmaceutical composition is typically a slow-release composition, providing slow-release of each of the calcium channel blockers and flavonolignan.

In one embodiment the slow release composition may comprises a unit dose of from 1 to 5000 mg of at least one flavanolignan (preferably 5 to 3000 mg such as 10 to 2000 mg or 50 to 1000 mg) and diltiazem in an amount of from 10 to 70 mg such as 10 to 50mg.

In a particularly preferred aspect we have found that the co-administration of calcium channel blocker (particularly diltiazem) that exerts its calcium blocking effect on mitochondria and has an antioxidant effect, and flavonolignan such as the flavonolignan extracted from blessed milk thistle (*Silybum marianum*), provide a significant improvement in the function and survival of liver cells under experimental conditions that mimic disease. Flavonolignan such as silymarin is frequently ineffectual in diseased cells and can be concentrated in the diseased liver to four or five times normal healthy levels thereby exacerbating the risk of significant side effects. We believe the co-administration of calcium channel blocker not only protects cell membranes but also facilitates at least partial regeneration of function in disease affected cells and improved metabolism of flavonolignans.
The action of silymarin as a hydrophilic antioxidant complements the antioxidant effect of diltiazem to protect both cell membranes and mitochondrial membranes of cells exposed to reactive oxygen species (ROS). These effects are complementary because the hydrophilic silymarin is predominately in the water phase of the cytosol where ROS are formed, whereas the lipophilic diltiazem is accumulated within the phospholipid of cell membranes where ROS produce damage by oxidizing phospholipid to lysophospholipid. Cell membrane damage caused by ROS increases the permeability of the cell and mitochondrial membranes to water and calcium thereby producing respectively cell swelling, hypoxia, and calcium overload.

Diltiazem as a calcium blocker inhibits calcium entry into mitochondria during conditions of calcium overload because it inhibits the calcium-gated calcium channel in the mitochondrial membrane which is activated when the intracellular calcium concentration rises. This action allows damaged or tired cells to continue to produce energy (ATP). This effect has a positive effect on liver cell function under conditions that resemble disease conditions.

While silymarin does not appear to have a direct effect on mitochondria other than contributing to the preservation of membranes on account of its antioxidant properties, the energy-protective effect of diltiazem on the mitochondria will produce an additional beneficial effect by enabling the cells to continue to metabolize the silymarin.

Preservation of the metabolism of silymarin, which is enabled by the direct mitochondrial effect of diltiazem, has two important clinical sequelae. First, it will help reduce the side effects of silymarin that may occur under conditions of liver disease if the metabolic capacity of the liver cells is reduced. Second, sustained metabolism of silymarin by the liver cells under disease conditions will help maintain the liver-selective delivery of silymarin because in addition to being administered in low dose, and in a slow-release formulation, a key feature of
liver-selective drug delivery is that the drug must have a short half-life produced by first-pass clearance by the liver.

In accordance with the present invention, we provide a method of treatment of a subject suffering from liver disease comprising the administration of (i) an oral slow-release formulation of a calcium channel blocker with antioxidant effects and (ii) at least one flavonolignan.

The invention also provides the use of (i) a calcium channel blocker with antioxidant effects and (ii) at least one flavonolignan, which is also an antioxidant, in preparation of a medicament for treatment of liver disease by co-administration thereof in one or more compositions for providing slow-release of calcium channel blocker.

The invention further provides a pharmaceutical composition for treatment or prevention of liver disease comprising (i) a calcium channel blocker with antioxidant effects and (ii) at least one flavonolignan which is also an antioxidant. The pharmaceutical composition is typically a slow-release composition that releases the active agents (lipophilic calcium channel blocker with antioxidant effects plus hydrophilic flavonolignan antioxidant, over a period of several hours, preferably 8 – 12 or more hours.

The liver-selective slow-release formulation is required because many of the drugs suitable for this treatment are vaso-active or have systemic effects that may be avoided if they are concentrated preferentially within the liver. The low dose, slow-release composition will preferably provide a delivery rate of calcium channel blocker sufficient to provide a clinical effective blood level in the portal vein and less than required to provide a clinically effective level in the peripheral circulation to thereby provide a delivery rate having a selective effect on the liver.
Thiamine may be administered in addition to the calcium channel blocker and flavonolignan separately or concurrently.

Thiamine, which is well known as a B vitamin acting within mitochondria as a mitochondrial protective agent, that is, as a cofactor of pyruvate dehydrogenase, is also a hydrophilic antioxidant. It is used in patients with nutritional deficiency and in some patients with viral hepatitis. The addition of thiamine to the calcium antagonist with antioxidant properties also exploits both the facilitator effect of the vitamin on energy production within mitochondria, and its own antioxidant effect within the cell.

**Detailed Description**

The liver is perfused at low pressure with venous blood. Therefore, more than any other organ in the body, it is at risk of hypoxia when cell swelling of any cause inhibits blood flow. Hypoxia of the liver is a feature of almost every form of non-malignant liver disease, and accounts for 50% or more of the pathology and impairment of function.

In inflammation, oxygen free radicals are produced by the inflammatory processes outside the cells. By contrast, in hypoxia, free radicals are produced inside the cells (as levels of NADPH rise). Both areas of free radical production are in the aqueous phase.

We have found that by using the two different types of antioxidants, that is, flavonolignans in combination with calcium channel blockers, a level of hepatoprotection is achieved, which is specifically enhanced when compared with a single antioxidant source. This is contrary to common belief that optimum protection is provided by high levels of an antioxidant.

Without wishing to be bound by theory, we believe that the enhanced effect from the combination may be due to the combination of intracellular antioxidant effects
of antioxidants from natural products, particularly flavonolignans, and intracellular antioxidant effects of calcium channel blockers which combat hypoxia free radicals within the cells. The combination provides a level of cellular stabilization of phospholipids which is significantly improved when compared with use of a single agent. Further while flavonolignins tend to accumulate in diseased liver cells their effectiveness as antioxidants is restored in the presence of the calcium chanel blocker. The metabolism of flavonolignins in diseased cells is also enhanced thereby reducing the risk of side effects of flavonolignins in subjects with liver disease.

The invention uses at least one antioxidant selected from flavonolignans. **Silibinin**, also known as **silybin**, is the major active constituent of **silymarin**, the mixture of flavonolignans extracted from blessed milk thistle (**Silybum marianum**). Where used herein the term flavonolignan includes silymarin and its active constituent silibinin whether in the form of the purified compound (believed to be 3,5,7-trihydroxy-2-(3-(3-hydroxy-4-methoxyphenyl)-2-(hydroxymethyl)-2,3-dihydrobenzo[b][1,4]dioxin-6-yl)chroman-4-one or pharmaceutically acceptable salts such as silibinin dihydrogen disuccinate disodium (trade name **Legalon SIL** a solution for injection), inclusion complexes such as β-cyclodextrin inclusion complexes and glycosides of silybin, which show better water solubility and even stronger hepatoprotective effect.

The invention allows the side effects associated with sylmarin to be reduced or generally eliminated allowing sustained use of the combination therapy without adverse effects over an extended period. Generally the composition will be administered at least once daily for at least ten consecutive days, preferably at least 20 and more preferably, at least 30 consecutive days.

In one embodiment the composition contains thiamine in a unit dosage form containing from 1 to 200mg thiamine, preferably 20 to 200mg thiamine. Patients which suffer from liver disease may have thiamine deficiency which may be
treated by administration of thiamine in an amount of from 1 to 5 mg per day. The use of relatively high doses of 20 to 200mg by contrast is well above the level required to treat vitamin deficiency. This dosage in the composition of the invention will provide a significant therapeutic effect believed to result from the antioxidant effects of the thiamine at relatively high dosage.

The preferred calcium channel blockers are lipophilic to ensure that they penetrate deep with cells and within membranes so that their therapeutic effect is located where free radicals are acting. By contrast, drugs acting within the cytosol or cellular milieu need to be more hydrophilic. Most calcium blocking drugs including verapamil, diltiazem, amlodipine, and nitrendipine have antioxidant properties as a component of a membrane stabilizing effect but the shorter half-life of diltiazem and verapamil makes these agents more suitable for formulation as a liver-selective membrane-stabilizing agent. The preferred agent is diltiazem administered as a slow-release formulation at doses of less than 70mg per day such as 10 to 70 mg per day or 20 to 70 mg per day and preferably less than 50 mg per day such as 10 to 50 mg per day. These doses are much lower that the doses of the drug used in the treatment of angina and hypertension. Diltiazem may be in the form of the hydrochloride salt or other pharmaceutically acceptable salt.

We have found that the combination of diltiazem and flavonolignans such as silymarin protected the cells against oxidative stress compared to either drug alone. Cell viability was statistically enhanced. Diltiazem, flavonolignan and the combination drug treatment were all associated with enhanced ATP levels in both cell types with the combination drug treatment showing a much greater protective effect. Bax and Bax mRNA levels were statistically reduced by drug treatment, again with the combination showing a much greater beneficial effect than either treatment alone. The combination of diltiazem and flavonolignan provided an additional hepatoprotective effect than either drug alone. The protective nature extends to reducing levels of Bax a pro-apoptotic protein. We have thus found
that the combination of the calcium channel blocker and flavonolignan not only has a significantly improved hepatoprotection when compared with each used separately but surprisingly we have found that the combination gives rise to a useful level of cell regeneration. As a result the treatment not only reduces the effects of the disease but also leads to cell regeneration and at least a partial reduction in the severity of the liver disease.

Diltiazem is preferably administered at a daily dose which is less than half (more preferably less than one third) of the dose prescribed for treatment of cardiovascular disease. The dose prescribed for treatment of cardiovascular disease is provided in medical texts relating to prescription drugs. We have referred, in particular, to the Australian Edition of MIMS Annual (2001) which lists the average optimum dose of diltiazem as 180 to 240 mg per day.

The preferred calcium channel blocker will be a membrane-stabilizing agent (or combination of agents) which has effects across several of the principal components of the membrane destructive process. The preferred calcium channel blocker will act as an intracellular and intramembranous antioxidant, limit calcium entry into the mitochondria, inhibit phospholipase activity, and to facilitate or maintain energy production by the cells. Furthermore, these actions preferably continue to operate as the cell pH falls during hypoxia. Together we believe these effects act to reduce cell swelling and hypoxia, and thence to improve liver function. We have found diltiazem to be particularly preferred on these bases.

Mitochondria in all cells have a calcium channel which is calcium-gated. The channel is activated as intracellular levels of calcium rise so that calcium is “shunted” into the mitochondria. This channel is different from the voltage-gated channel of excitable tissues (muscle and arteries), but is inhibited by almost all calcium blockers (calcium antagonists).
Patients with liver disease frequently have low normal blood pressure, and there is a need to ensure that treatment does not lower blood pressure to levels that may contribute to fatigue and impairment of liver function.

Liver-selective drug delivery of a short-acting calcium blocker is one solution to the risk of hypotension and fatigue, but diltiazem as a benzothiazine, and in contrast to verapamil and the dihydropyridines, results in very little blood-pressure lowering effect in normotensive patients and is therefore preferred over other calcium antagonists. Subcellular studies have shown that protective effect of diltiazem appears to occur at concentrations of diltiazem that are much higher than the those used in whole cell studies or than plasma concentrations of the drug observed after oral administration. However, the lipophilic nature of the drug means that membranes absorb it achieving a concentration gradient of 100:1 or more between the membrane and plasma. This also explains why the mitochondrial calcium blocking effect can take 2 hours or more to develop after the drug is applied to cell cultures.

Diltiazem, which is cis-\((+)-3-(acetyloxy)-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5H)-one\), referred to herein as "diltiazem", is a benzothiazine derivative possessing calcium antagonist activity. Diltiazem has heretofore been used clinically to block the influx of calcium ions in smooth and cardiac muscle and thus exert potent cardiovascular effect. Diltiazem has been shown to be useful in alleviating symptoms of chronic heart disease, particularly angina pectoris and myocardial ischemia and hypertension, while displaying a low incidence of side effects. In these uses diltiazem is available as diltiazem hydrochloride in tablet form in strengths of 30, 60, 90 and 120 mg. and in capsule form in strengths of 60, 90, 120, 180, 240 and 300 mg. Diltiazem is also available in injectable form at a concentration of 5 mg/ml.

Diltiazem therapy for treatment of cardiovascular disease typically starts with 30 mg. administered 4 times daily. The dosage is gradually increased to 180 to 360
mg/day, given in divided doses three or four times daily, at one- to two- day intervals until an optimum response is obtained. Diltiazem is extensively metabolized by the liver. According to professional use information issued by Marion Merrell Dow Inc., diltiazem in CARDIZEM.RTM. brand tablets is absorbed to about 80% and is subject to an extensive first-pass effect, giving an absolute bioavailability, compared to intravenous administration, of about 40%. Single oral doses of 30 to 120 mg. of CARDIZEM.RTM. diltiazem tablets result in peak plasma levels two to three hours after administration. Detectable plasma levels occur within 30 to 60 minutes after administration indicating that CARDIZEM.RTM. diltiazem tablets are readily absorbed. The plasma elimination half-life following single or multiple administration is approximately 3.5 hours. Therapeutic blood levels of CARDIZEM.RTM. diltiazem tablets appear to be in the range of 50 to 200 ng./ml.

In contrast with prior art formulation of calcium channel blockers the first pass clearance of drugs such as diltiazem, which is viewed as an impediment to effective treatment of cardiovascular disease, becomes a virtue in treating liver disease as it allows the clinical effect of the drug to be confined to the liver. Accordingly it is particularly preferred that the calcium channel blocker be present in a low dose and as a slow-release formulation to provide a clinical effective blood level in the portal vein and a dose less than required to provide a clinically effective level in the peripheral circulation. The method and composition of the invention thus provide a delivery rate having a selective effect on the liver.

This method of hepatic protection using diltiazem in combination with flavonolignans and optionally also thiamine, applies to any disease state of the liver in which the cell membranes have been damaged either primarily or secondarily by oxidising agents or oxidising processes. The use of a liver-selective formulation of these membrane-stabilizing agents is complementary to the treatment of the primary disease.
The direct mitochondrial effect of diltiazem, which enables preservation of liver function under disease conditions also has a clinically important effect on the metabolic clearance of silymarin. First, it will help reduce the side effects of silymarin that may occur under conditions of liver disease if the metabolic capacity of the liver cells is reduced. Second, sustained metabolism of silymarin by the liver cells under disease conditions will help maintain the liver-selective delivery of silymarin because in addition to being administered in low dose, and in a slow-release formulation, a key feature of liver-selective drug delivery is that the drug must have a short half-life produced by first-pass clearance by the liver.

When appropriate these liver-protective agents may be co-prescribed or co-formulated with therapeutic agents used in the primary management of the disease, such as ribavirin or other orally-administered antiviral agent in the management of hepatitis C.

The invention thus also provides a method of treatment or prevention of liver disease by co-administering or co-formulating together with other active agents such as are used to treat portal hypertension or viral hepatitis, the liver protective therapy detailed in this invention that includes a liver-selective formulation of a calcium channel blocker and antioxidant such as diltiazem with at least one flavonolignan and optionally also a further antioxidant agent such as thiamine which is relatively hydrophilic compared with the calcium channel blocker.

The two components of the composition, that is, the calcium channel blocker plus the flavonolignan may be co-formulated or may be administered in a separate composition. The agents may be administered by the same route (particularly oral administration) or may be administered by different routes. For example, one active agent may be administered intravenously or parenterally and the calcium channel blocker administered orally.
In the method and composition of the invention thiamine may, when used, be in the form of the salt from such a hydrochloride salt or other pharmaceutically acceptable derivative. Patients suffering from toxic hepatitis have an impaired ability to absorb thiamine despite having low thiamine concentration in the blood that are often difficult to measure. The production of energy in the mitochondria is thus severely compromised in cells of a diseased liver.

In one particularly preferred embodiment of the invention at least one agent selected from agents used to treat portal hypertension or an antiviral agent, is co-formulated with calcium channel blocker, flavonolignan and optionally thiamine.

In a further embodiment the invention provides a method of treatment or prevention of toxic hepatitis (for example alcoholic hepatitis, but also including fatty liver) comprising co-administration of the liver-selective formulation of a calcium blocker and thiamine. It is particularly advantageous if thiamine, which is hydrophilic, is co-formulated with the calcium channel blocker component. Accordingly in the preferred embodiment we provide a composition for treatment of toxic hepatitis comprising a liver selective formulation of a calcium channel blocker such as diltiazem and a flavonolignan particularly silymarin, silibinin and pharmaceutically acceptable antioxidant derivatives thereof.

The preferred calcium channel blocker is diltiazem in an amount of from 20 to 70 mg/day and more preferably 25 to 50 mg/day.

The daily dosage of flavonolignan such as silibinin or silymarin is in the range of from 1 to 5000mg/day and preferably from 10 to 2000mg/day.

In one embodiment of the invention the ratio of the daily dosage of diltiazem:flavonolignan is in the range of from 50:1 to 1:120 and more preferably from 2:1 to 1:120.
When thiamine is used it is preferably present in an amount to provide delivery of from 1 to 200 mg per day and more preferably 20 to 200 mg per day.

Throughout the description and claims of the specification the word “comprise” and variations of the word, such as “comprising” and “comprises”, is not intended to exclude other additives, components, integers or steps.

The use of the vitamin thiamine adds a complementary mitochondrial effect in the mitochondria where as a co-factor of pyruvate dehydrogenase, that facilitates energy production. This therapy may be used to protect the liver and maintain hepatic function when the liver has been damaged by any form of chronic non-malignant disease in which the cell membranes have been damaged either by primary or secondary processes such that the liver cells swell sufficient to impede portal venous blood flow and cause hypoxia of the liver.

Diseases in which the treatment of the present invention may have application include all forms of viral hepatitis (including hepatitis B and C), alcoholic liver disease, cirrhosis of the liver, toxic hepatitis, autoimmune hepatitis, fatty liver, damage to the liver sustained during chemotherapy or radiotherapy, and ageing of the liver.

Progressive deterioration of the structure and function of cell membranes within the liver is a key component of almost all forms of chronic, non-malignant liver disease. It contributes to the overall impairment of liver function, leads to cell death, and complements or enhances the effects of the primary disease processes.

The concept of liver-selective drug delivery requires that a drug with a short half-life be administered as low dose and as a slow-release formulation. This is very different from an enteric coating and provides a release over a period of preferably at least 8 hours and up to 24 hours as the drug descends through the
small intestine. After crossing the gastrointestinal wall, the drug reaches the relatively small volume of the portal venous system and is carried to the liver. Here a significant portion is removed from the circulation by metabolism with the remainder passing into the much larger volume systemic circulation. In this way, a stable concentration gradient is achieved where the concentration of the drug is up to 5 or more times higher in the liver and portal circulation than in the systemic circulation. The achieved concentration gradient may be higher in cirrhosis or other conditions with sluggish portal circulation, however, this effect may be offset by the development of significant collateral vessels between the portal and systemic circulations.

In many therapeutic situations it is desirable to limit delivery of a drug to the target organ. This is particularly important when a drug has side effects related to other pharmacological properties. In the case of membrane-stabilizing agents that are also calcium channel blockers (and potent vasodilators), their presentation as liver-selective therapy avoids or minimises the risk of unwanted vasodilator and cardiac effects thereby increasing tolerance and acceptability. Liver-selective delivery also reduces the total daily dose of a drug required to 20 – 25% of the full systemic dose that would normally be required if the drug was being administered to achieve a systemic effect.

In the case of diltiazem, where the doses required for systemic effects used in the treatment of angina pectoris and hypertension are generally in the range of 120 – 360 mg/day, a dose of 50mg or less per day of diltiazem will retain a therapeutic effect in the liver but not the rest of the body.

A membrane-stabilizing agent with these properties and including diltiazem formulated for liver-selective delivery with or without thiamine can be used to protect the liver in:

- Viral hepatitis including Hepatitis B, and C, and other forms.
- Alcoholic hepatitis
- Cirrhosis of the liver
- Portal hypertension
- Fatty liver including Non-alcoholic Steatohepatitis (NASH)
- Other forms of non-malignant liver disease caused by toxins, drugs, and abnormal immune states.
- Liver dysfunction during chemotherapy.
- Liver dysfunction after radiotherapy
- The ageing liver.

In the case of viral hepatitis, the immediate cause of damage to liver cell membranes is the body's immune response to the presence of the virus. (Loguerco and Federico, 2003). This leads to generation of free radicals with damage to cell membranes on the surface and the inside of the cell (Jain et al, 2002). It follows that while the use of interferon with or without added ribavirin (or related molecules) is necessary antiviral treatment, there is a need to protect or reverse damage done to the liver cell by the immune processes. Therefore a liver-selective membrane-stabilizing agent with both antioxidant and mitochondrial effects is complementary to the primary treatment. Furthermore, in those communities of people who cannot afford expensive virucidal therapy, treatment with a liver-selective membrane-stabilizing agent such as diltiazem or related molecules that can minimise or contain symptoms becomes an affordable alternative. A liver-selective membrane-stabilizing agent with or without added thiamine can therefore be prescribed as monotherapy, be co-prescribed with interferon and ribavirin or with other appropriate antiviral therapy, or be co-formulated with ribavirin or other orally-administered component of the antiviral therapy so that both or all drugs are administered together as a liver-selective formulation.

Treatment with thiamine (Vitamin B1) has been used in management of alcoholic liver disease because patients with this disease frequently have a nutritional deficiency of the vitamin. Thiamine acts as a co-enzyme for several reactions
that cleave carbon-carbon bonds including key metabolic processes within the hepatic mitochondria (Wilson, 1998). In addition to correction of nutritional deficiency, treatment with thiamine may improve drug-induced mitochondrial damage in patients with hepatitis B and C (Kontorinis and Dieterich, 2003).

Other workers have shown that thiamine deficiency is common in patients with cirrhosis caused by both alcoholic liver disease and Hepatitis C, but not in Hepatitis C without cirrhosis (Levy and colleagues, 2002). However, thiamine has been shown to improve liver function in patients with Hepatitis B (Wallace and Weeks, 2001). These workers have suggested that the vitamin may have a protective effect on mitochondrial function in patients with viral hepatitis. Another key component of the protective affect of thiamine is its antioxidant properties, which appear to be active in the cytosol on account of its hydrophilic profile.

Evidence is accruing that treatment with thiamine (vitamin) may be helpful in patients with viral hepatitis as a mitochondrial protective agent (Wallace AE and Weeks WB, 2001), and particularly in those patients with cirrhosis caused by hepatitis C (Levy et al, 2002). Co-prescription or co-formulation of a liver-selective membrane-stabilizing agent and thiamine may be helpful in these patients. In particular we believe the complementary energy-facilitating role of thiamine and its antioxidant effect together with the membrane stabilizing agent provide a significant advantage.

In patients with portal hypertension and cirrhosis, the immediate treatment is correction of the underlying causative liver disease, and then reduction of the vascular resistance to portal venous flow, or immediate reduction of portal venous flow with propranolol or other non-selective beta-adrenergic antagonist. Therefore the composition with both antioxidant and mitochondrial calcium inhibition is complementary to this primary treatment. The composition may be co-prescribed with propranolol.
In other forms of liver disease including toxic hepatitis, immune hepatitis, and damage to the liver during the course of systemic chemotherapy, attack of the cell membranes of the liver cell by oxidising agents and free radicals from both within and without the cell is the principle pathological mechanism of the disease. This in turns causes the cells to swell inducing hypoxia with a second stage of free radical production throughout the cell and the mitochondria. Radiotherapy also generates free radicals. While this may be a key mechanism targeted at organs with cancer when the liver is irradiated either necessarily or intentionally, the normal physiology with low pressure perfusion by portal venous blood makes the organ particularly at risk of hypoxia and deterioration of function. Therefore the coadministration of the calcium channel blocker and flavonolignan is complementary to drug or toxin withdrawal, management of the immune disease, treatment with systemic chemotherapy, or after radiotherapy.

In the ageing liver, there is a reduction of the oxidising properties of the liver such that the liver is less able to protect itself from naturally occurring oxidising effects in the diet. These changes, which involve both mitochondrial and cytoplasmic function, make the liver more at risk of cell swelling, modest hypoxia and impaired function. Therefore the coadministration of the calcium channel blocker and flavonolignan is useful treatment for management of the ageing liver to help maintain hepatic function in older patients with any degree of impaired hepatic function.

Formulation for slow-release

There are many techniques to effect slow-release of an active pharmaceutical agent from an orally-administered formulation. These methods may include techniques designed to delay the disintegration of a capsule, tablet, or other vehicle, techniques designed to delay the solubility of a capsule, tablet or other vehicle, and techniques in which an active agent may be bound to a polymer or other large molecule such that absorption can not take place until the substance has been released from the polymer or other large molecule. The means of
achieving these different methods of slow-release are varied and include well-known older methods, such as layers of shellac coating, and more modern techniques using synthetic and cellulose polymers or providing a membrane coating with at least one pore.

The dosage forms according to the present invention may be controlled-release dosage forms. The mechanism of release of these dosage forms can be controlled by diffusion and or erosion. In some embodiments, the formulation comprises polymer-coated multiparticulates, polymer-coated tablets or minitablets, or hydrophilic matrix tablets.

A slow-release formulation of a membrane-stabilizing drug designed to act as a hepatoprotective agent may be designed to release the drug over a period of about 6 to about 24 hours following administration, thereby permitting once-a-day administration and providing a sustained exposure of the drug to the liver. In some embodiments, formulations releasing the drug over extended periods of time may have more than one timed-release component to affect time coverage.

The present invention relates to the finding that a protective effect of low-doses of membrane-stabilizing agents (including diltiazem at doses of less than 50 mg/day) administered as a slow-release formulation, is mediated by a direct protective effect on the liver itself rather than on the vasculature, that the drug acts as a lipophilic antioxidant to penetrate cells and protect both cell membranes and mitochondria from the damaging effects of phospholipid oxygenation, and that it acts as a mitochondrial calcium antagonist.

The slow-release composition may be of a type previously reported for treatment of cardiovascular disease with the exception that the dose of diltiazem is reduced to within the range of from 20 to 70 mg per day. As discussed above the slow-release composition will preferably also contain thiamine in an amount of from 1 to 5 mg per day. The composition may be designed for once daily administration,
twice daily administration or more often but once daily administration is particularly preferred.

U.S. Pat. Nos. 4,721,619, 4,891,230, 4,917,899 and 5,219,621 disclose diltiazem formulations that purport to require administration once every twelve hours (i.e., twice a day). U.S. Pat. Nos. 4,894,240 and 5,002,776 disclose diltiazem formulations that purport to require administration once every 24 hours (i.e., once a day). To obtain the dissolution profiles disclosed in these patents, the formulations disclosed require a multi-layer membrane that coats the central core and an organic acid in the active core and/or in the multi-layer membrane. Suitable organic acids disclosed in these patents are adipic acid, ascorbic acid, citric acid, fumaric acid, malic acid, succinic acid and tartaric acid. According to professional use information issued by Marion Merrell Dow Inc., the CARDIZEM.RTM. CD diltiazem capsule is a sustained release diltiazem capsule containing 120, 180, 240 or 300 mg. diltiazem hydrochloride with a suggested dosage of one capsule a day. Similarly, to obtain a 24-hour diltiazem release profile, the pellets in the CARDIZEM.RTM. CD diltiazem capsule include fumaric acid, an organic acid, and a multi-layer membrane that coats the central core. According to the aforementioned patents, the pellets must be dried for a number of hours during and after the coating process.

Another approach which may be adapted for use in the process of the invention is described in US Patent 5 834 024 (Heinicke et al) which uses a combination short lag and long lag pellets of diltiazem to provide a uniform release of diltiazem over a 24 hour period.

In one embodiment of the composition of the invention the slow-release composition comprises a core containing diltiazem and one or more polymeric coatings. The core may and preferably will contain the thiamine component. The core may be formed on a seed of, for example, an inert material such as a sugar sphere. Pharmacologically acceptable binders such as hydroxypropyl cellulose
may be used in the core. The one or more coatings may comprise a polymer which is permeable to diltiazem and water and a polymer which is relatively less permeable to diltiazem and water.

An example of a diltiazem permeable polymer is the cationic polymer synthesized from acrylic and methacrylic acid ester with a low content of quaternary ammonium groups, known as EUDRAGIT RL (manufactured by Rohm Pharma GmbH) ethylcellulose, cellulose acetate, cellulose propionate (lower, medium or higher molecular weight), cellulose acetate propionate, cellulose acetate butyrate, cellulose acetate phthalate, cellulose triacetate, poly(methyl methacrylate), poly(ethyl methacrylate), poly(butyl methacrylate), poly(isobutyl methacrylate), poly(hexyl methacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate), poly(ethylene), poly(ethylene) low density, poly(ethylene) high density, poly(propylene), poly(ethylene oxide), poly(ethylene terephthalate), poly(vinyl isobutyl ether), poly(vinyl acetate), poly(vinyl chloride) or polyurethane or a mixture of any two or more of these. Suitable naturally occurring polymers or resins that are less permeable to water and diltiazem include shellac, chitosan, gumjuniper or a mixture of two or more of these.

Substances that can be used that are less permeable to diltiazem and water include a cationic polymer known as EUDRAGIT RS (manufactured by Rohm Pharma GmbH). EUDRAGIT RS is less permeable than EUDRAGIT RL because EUDRAGIT RS has fewer ammonium groups) ethylcellulose, cellulose acetate, cellulose propionate (lower, medium or higher molecular weight), cellulose acetate propionate, cellulose acetate butyrate, cellulose acetate phthalate, cellulose triacetate, poly(methyl methacrylate), poly(ethyl methacrylate), poly(butyl methacrylate), poly(isobutyl methacrylate), poly(hexyl methacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate),
poly(octadecyl acrylate), poly(ethylene), poly(ethylene) low density, poly(ethylene) high density, poly(propylene), poly(ethylene oxide), poly(ethylene terephthalate), poly(vinyl isobutyl ether), poly(vinyl acetate), poly(vinyl chloride) or polyurethane or a mixture of any two or more of these. Suitable naturally occurring polymers or resins that are less permeable to water and diltiazem include shellac, chitosan, gumjuniper or a mixture of two or more of these.

In addition to the polymers, the coating layer includes a lubricant and a wetting agent. Preferably the lubricant is talc and the wetting agent is sodium lauryl sulfate.

Suitable alternatives for sodium lauryl sulfate may include agents such as acacia, benzalkonium chloride, cetomacrogol emulsifying wax, cetostearyl alcohol, cetyl alcohol, cholesterol, diethanolamine, docusate sodium, sodium stearate, emulsifying wax, glyceryl monostearate, hydroxypropyl cellulose, lanolin alcohols, lecithin, mineral oil, monoethanolamine, poloxamer, polyoxyethylene alkyl ethers, polyoxyethylene castor oil derivatives, polyoxyethylene sorbitan fatty acid esters, polyoxyethylene stearates, propylene glycol alginate, sorbitan esters, stearyl alcohol and triethanolamine, or a mixture of any two or more of the foregoing.

Suitable alternatives for talc that may be included in the coating are calcium stearate, colloidal silicon dioxide, glycerine, magnesium stearate, mineral oil, polyethylene glycol, and zinc stearate, aluminium stearate or a mixture of any two or more of the foregoing.

A plasticizing agent is preferably included in the coating to improve the elasticity and the stability of the polymer film and to prevent changes in the polymer permeability over prolonged storage. Such changes could affect the drug release rate. Suitable conventional plasticizing agents include acetylated monoglycerides, acetyltributylcitrate, acetyltributyl citrate, castor oil, citric acid esters, dibutyl phthalate, dibutylysebacate, diethyloxalate, diethyl malate,
diethylfumarate, diethylphthalate, diethylsuccinate, diethylmalonate, diethyltartarate, dimethylphthalate, glycerine, glycerol, glyceryl triacetate, glyceryltributyrate, mineral oil and lanolin alcohols, petrolatum and lanolin alcohols, phthalic acid esters, polyethylene glycols, propylene glycol, rape oil, sesame oil, triacetin, tributyl citrate, triethyl citrate, and triethyl acetyl citrate, or a mixture of any two or more of the foregoing. Triethyl citrate is the presently preferred plasticizing agent.

Alternatively or in addition the core or coating may comprise an organic acid such as adipic acid, ascorbic acid, citric acid, fumaric acid, malic acid, succinic acid, tartaric acid and fumaric acid.

An alternative method of controlled release is to use membrane coating about a core comprising the active agents which membrane has at least one pore therein. Such an arrangement is described by Chen et al in US 6,866,866 for providing slow-release of metformin for a once a day dose. Chen et al also describe a slow-release formulation for diltiazem in US Patent 6,524,620 which may be utilised with the low dose composition of the invention for treatment of liver disease.

The invention will now be described with reference to the following examples. It is to be understood that the examples are provided by way of illustration of the invention and that they are in no way limiting to the scope of the invention.

**EXAMPLES**

While silymarin has a complex composition, other workers have shown that it is possible to formulate this herbal agent in erodable matrix based on systems based on glyceryl monostearate and polyethylene glycol 6000 or poloxamer 188 (Cheng et al 2007.) It follows that silymarin and other herbal antioxidants can be co-formulated with diltiazem using many of the standard techniques for preparation of slow-release formulation. The claims of this invention relate to the
concept of co-formulation a herbal antioxidant with low-dose diltiazem in a slow-release formulation rather than to the specific chemical nature of the formulation used.

5 Comparative Example
This Example Compares the effect of diltiazem, silymarin and thiamine when separately administered in protecting hepatocytes from reactive oxygen species

Methods
10 Isolated rat liver mitochondria were incubated with low concentrations of Ca$^{2+}$ to partly uncouple oxidative phosphorylation, and oxygen consumption and acceptor control ratios measured. The concentration of diltiazem in the extra mitochondrial medium was measured using LC-mass spectrometry. Rat hepatocytes isolated by collagenase digestion and cultured on coverslips and in multiwell plates were incubated with H$_2$O$_2$ (to generate reactive oxygen species) and cell viability was assessed by measuring lactate dehydrogenase release using a Cobas Fara autoanalyser.

Results
20 In mitochondria using succinate as a substrate, Ca$^{2+}$ (170 µM) decreased the acceptor control ratio from 3.3 to 1.9. D(+) diltiazem pre-incubated for 5 min at concentrations of 210, 410 and 790 µM increased the acceptor control ratio to 2.0, 2.3 and 2.6, respectively. Addition of thiamine, a co-factor for mitochondrial pyruvate and α-ketoglutarate dehydrogenases, did not affect the actions of diltiazem. Measurement of the concentration of diltiazem in the extra mitochondrial medium at the end of the experiment showed that a substantial amount of diltiazem was partitioned into the mitochondrial membranes. The measured concentrations of diltiazem were 142 ± 14 (5), 275 ± 10 (6) and 553 ± 38 (3) µM at 205, 410 and 790 µM added diltiazem, respectively. Incubation of isolated rat hepatocytes with 0.5 mM H$_2$O$_2$ for 24 h induced 87% cell death. When hepatocytes were pre-treated for 2 h with 1 µM diltiazem, 1 µM diltiazem +
50 μM thiamine, 50 μM thiamine alone, 50 μM silymarin, or 100 μM silymarin, the proportion of dead cells was 5, 3, 87, 88 and 90%, respectively. Thus diltiazem, but not low concentrations of silymarin, substantially reduced H₂O₂-induced hepatocyte death.

Conclusions
It is concluded that diltiazem, at low concentrations, is effective in protecting hepatocytes from damage induced by reactive oxygen species. This may, in part, be due to the actions of diltiazem in modulating Ca²⁺ movement and preserving the capacity of mitochondria to synthesise ATP. Under the conditions employed, diltiazem was considerably more effective than low concentrations of silymarin. These results suggest that diltiazem could play a valuable role in protecting hepatocytes from damage induced by reactive oxygen species and other agents but that Silymarin has a relatively small effect.

Example 1
This Example compares the response of hepatocytes under oxidative stress to the actions of diltiazem, silymarin and a combination of diltiazem and silymarin.

Materials and Methods
All chemicals including d-cis diltiazem, silymarin, and dichlorofluorescein diacetate (DCFH₂-DA) were purchased from Sigma Chemical (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin, streptomycin, were purchased from Gibco/BRL. MTT (cell proliferation) and ATPase kits were purchased from Sigma (St. Louis, MO). Human hepatoma cell lines Chang and PLC/PRF/5 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD).

Cell Culture and Drug Treatment
Chang and PLC/PRF/5 hepatoma cells were maintained in 25 cm² culture flasks supplemented with DMEM containing 10% FBS (DF-10), penicillin and
streptomycin (50 units/ml) at 37°C in a humidified atmosphere of 95% air and 5% CO₂ for at least 24 hours. Cells were plated onto culture plates (100 mm²) and exposed to DF-10 media and drug treatment for 24 hours for Western blot and RT-PCR studies. For DCF and MTT assays cells were subcultured onto 96-well black plates (Corning, NY, USA) and allowed to attach for 8 hours prior to any drug treatment. Following plate confluence, cells were exposed to DF-10 media containing drugs for 24 hours. Control cells were incubated without any drug.

**DCF Assay**

The conversion of the non-fluorescent 2',7'-dichlorodihydrofluorescein (DCFH) to the highly fluorescent dichlorofluorescein (DCF) by reactive oxygen products, such as lipid peroxides, forms the basis of the assay. The active intracellular 2',7'-dichlorodihydrofluorescein (DCFH) form of DCFH₂-DA was prepared as described by Tollefson et al. ¹⁴. Briefly, the diacetate moiety of DCFH₂-DA was cleaved by incubating 100 μL stock DCFH₂-DA in the presence of 200 μL methanol and 100 μL 2N NaOH at room temperature in the dark. After 2 hours the resulting DCFH solution was diluted with Ca/Mg-PBS to achieve a final probe concentration of 20 μM with the pH adjusted to 7.4 immediately before use.

**Oxidative stress induced by H₂O₂**

Chang and PLC cells were cultured in a 96-well culture plates (Costar# 3603, Corning Incorporated) at a cell density of 25,000 cells per well. After overnight incubation in DMEM-10% FBS, cultures were washed twice with PBS and then incubated with 10 μM H₂DCFDA in 95% air/5% CO₂ for 30 min at 37°C. Extracellular H₂DCFDA was removed by washing the culture twice with warm PBS. Cellular oxidative stress was induced by incubating cells with 400 μM H₂O₂ in PBS containing Ca⁺⁺ and Mg⁺⁺ for 20 min at 37°C in the dark. Negative controls were performed using the same conditions but without H₂O₂. Cellular fluorescence intensity of emitted DCF signal was immediately measured using a BMG (Durham, NC, USA) Fluostar Galaxy fluorescence plate reader (485 nm excitation wavelength/520 nm emission wavelength), equipped with excitation
and emission probes directed to the bottom of the plate. Mean fluorescence intensity was calculated from triplicate cultures of control and drug treated cells. Results were expressed in arbitrary fluorescence units (AFU).

5 **Cell viability/Cell proliferation: MTT Assay**
The MTT assay was used to assess cell growth and viability. Living cells reduce MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) to purple formazan by functional mitochondria. Assay conditions were as outlined by the manufacturer (Sigma, St. Louis, MO). Briefly, Chang and PLC cells were cultured in 96-well plates at a concentration of $3 \times 10^4$ cells/well. Following exposure to diltiazem and/or silymarin for 24 hours and $H_2O_2$ (400μM, 20 minute) treatment, 10μl of the MTT solution (10% of the culture volume) was added to each well. Cells were incubated with the MTT solution for an additional 3.5 hours at which time the culture media was removed and the MTT solvent added to the wells (100 μl). Absorbance of the coloured solution was quantitated at 570 nm by using a spectra Max 190 (Molecular Devices) plate reader.

**Determination of cellular ATP levels**
A bioluminescent assay was employed for the determination of total cellular ATP levels. Procedural details were followed as outlined by the manufacturer (Sigma, St. Louis, MO).

**Bax Western blot**
Proteins (20 μg) were mixed with 4X gel loading buffer (4X = 250 mmol/L Tris-HCl, pH 6.8, 8 % SDS, 20 % glycerol, 0.2 % bromophenol blue and 5 % β-mercaptoethanol), separated on 15 % sodium dodecyl sulfate-polyacrylamide (SDS-polyacrylamide) gel under reducing conditions, and transferred onto Nitroplus-2000 membrane (Micron Separations Inc. Westborough, MA). Nonspecific antibody binding was blocked by pre-incubation of the membranes in dry skim milk in 1X Tris-buffered-saline-Tween (TBS-T, 2.42 g Tris base, 29.25 g NaCl, 0.5 ml Tween 20 per liter) for 1 hour at room temperature. Membranes
were then incubated with primary antibody in 1X TBS-T containing 5% skim milk at 4°C overnight. The titration of primary antibody was 1:1000 for rabbit anti-Bax and 1:3000 for monoclonal mouse anti-β-Actin. Membranes were subsequently incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit antibody for and Bax and anti-mouse antibody for β-Actin (1:1000 dilution). The antigen-antibody complexes were detected by enhanced chemiluminescence (ECL system, Amersham). The optical density (OD) values of each target protein band were determined by using NIH Imaging software. Protein loading variation was corrected by normalization of β-Actin.

Bax RT-PCR

TRIzol LS reagent was employed to extract total RNA of the Chang cells as described in the manufacturer’s manual. The first-strand cDNA was synthesized by an Advantage RT-for-PCR kit. Briefly, 1 µg of total RNA was dissolved in diethylpyrocarbonate-treated doubled-distilled water (ddH2O) to achieve a final volume of 12.5 µl. Subsequently, 1 µl oligo(dT) primer, 4 µl of 5 x reaction buffer, 1 µl of 10 mmol/l dNTP mix, 0.5 µl recombinant RNase inhibitor, and 1 µl Moloney murine leukemia virus reverse transcriptase were added and incubated for 1 h at 42°C. At the end of the reverse transcription procedure the reaction mixture was heated to 94°C for 5 min and brought up to a final volume of 100 µl with ddH2O. PCR was performed by using the Advantage PCR kit, polymerase mix, and oligonucleotides synthesized by GIBCO-BRL (Burlington, ON). Gene-specific PCR primers for Bax were purchased from Sigma (St. Louis, MO). The oligonucleotide primers were 5'-GTT TCA TCC AGT ATC GAG CAG CAG-3' (sense), 5'-CAT CTT CTT CCA GAT GGT GA-3' (antisense). Product length was 487bp. The specific rat GAPDH amplimers were from Clontech (5507-3) with an expected GAPDH size of 986 base pairs. PCR amplification was carried out in 30 cycles of denaturation at 94°C for 45 seconds, annealing at 53°C (Bax) and 60°C (GAPDH) for 45 seconds, elongation at 72°C for 120 seconds with an additional 7-minute final extension at 72°C using an Eppendorf MasterCycler
(Eppendorf, Westbury, NY). The PCR product was analyzed using a 1.2% agarose gel. Identity of PCR products was confirmed by sequencing at the DNA-sequencing facility of the Manitoba Institute of Cell Biology (Winnipeg, Canada).

Statistical Analyses

Data are presented as mean ± SEM. The n value refers to the number of replicates performed for each study. Data were analyzed using one-way ANOVA with Student-Newman-Keuls post hoc test taking p<0.05 as the level for significance (statistical significance set at p<0.05).

The results are discussed with reference to the attached drawings. In the drawings:

Figure 1. DCF fluorescence of PLC (Figure 1a) and Chang (Figure 1b) cells in oxidative stress induced by hydrogen peroxide. Cells were cultured in 96-well plates. H$_2$DCFDA (10 µM) was loaded onto cells for 30 min. Cells were subsequently exposed to 400 µM H$_2$O$_2$ for 20 min. Cellular fluorescence in each well was measured and immediately recorded. Data are represented as mean ± SEM; n = 7; ** p< 0.01, ***p<0.001; one tail distribution.

Figure 2. Cell growth as assessed by the MTT assay for PLC (Figure 2a) Chang (Figure 2b) cells following diltiazem and/or silymarin. Cells were cultured in 96-well plates. Data represent mean±SEM; n = 7; ** p< 0.01, ***p<0.001; one tail distribution.

Figure 3. Cellular ATP levels in control (no oxidative stress treatment) in PLC cells (Figure 3a) and Chang cells (Figure 3b) as assessed by the bioluminescent assay. Data represent mean±SEM; n = 7.
Figure 4. Cellular ATP levels in PLC cells as assessed by the bioluminescent assay. Data represent mean±SEM; n = 7; ** p< 0.01, ***p<0.001; one tail distribution.

Figure 5. Expression of Bax in control and drug treatment Chang cells. Blank contained serum-free media cells while control contained serum-containing media following H$_2$O$_2$ treatment. Anti-apoptotic effect of diltiazem, silymarin, and the combination drug treatment in Chang cells. Apoptosis was induced by H$_2$O$_2$ treatment. Bax activity was used as the marker of apoptosis.

Figure 6. The relative Bax expression in Chang cells calculated by normalizing Bax optical density against actin. Data represent mean±SEM; n = 7; * p< 0.05, ** p< 0.01, ***p<0.001; one tail distribution.

Figure 7. RT-PCR image of Bax and loading control GAPDH in control and drug treated Chang cells.

**Intracellular Free Radical Levels**

Cellular free radical levels were assessed using the dichlorofluorescein (DCF) assay. Figure 1 shows DCF fluorescence from H$_2$O$_2$ induced oxidative stress treated with diltiazem (2.5 and 10 μM), silymarin (10 and 1000 μg/L) or the combination diltiazem (2.5 μM or 10μM) and silymarin (1000 μg/L) for PLC cells (top) or diltiazem (2.5 μM) and silymarin (1000 μg/L) or diltiazem (10 μM) and silymarin (10 μg/L) for Chang cells (bottom). Both diltiazem and silymarin statistically reduced the free radical levels in both PLC and Chang cells (p<0.05). DCF fluorescence was not statistical different between diltiazem and silymarin treatment. The combination of diltiazem and silymarin further reduced the DCF fluorescence from that of either silymarin or diltiazem alone as shown in Figure 1 (p<0.001).

**Cell Proliferation**
Cellular viability and proliferation were assessed using the MTT kit from Sigma. Figure 2 shows that both diltiazem and silymarin statistically increased cellular proliferation from control (no drug treatment) levels (p<0.001). The addition of silymarin (1000 µg/L) to 2.5 µM diltiazem further enhanced cell proliferation (p<0.001). Increasing the diltiazem concentration to 10 µM did not provide any further increase in proliferation than the use of 2.5 µM.

**Cellular ATP levels**

To understand the protective nature of diltiazem, silymarin, and the combination of diltiazem and silymarin we investigated the cellular ATP levels following drug treatment. Figure 3 shows that in the control group (no oxidative stress) drug treatment did not significantly affect cellular ATP levels. However, as shown in Figure 4 following oxidative stress (H₂O₂ treatment) ATP levels were significantly (p<0.001) higher than control (no drug treatment). There was no significant difference between 2.5 µM diltiazem and 10 µM diltiazem. Silymarin by itself also significantly increased the ATP levels (p<0.01). The addition of silymarin to diltiazem further enhanced the ATP levels than did diltiazem alone (p<0.001) or silymarin alone (p<0.01). There was no statistical enhancement on ATP levels when the diltiazem dose was increased (2.5 µM to 10 µM) and silymarin (1000 µg/L).

**Bax levels**

To further understand the protective nature of diltiazem and silymarin we investigated whether drug treatment affected the pro-apoptotic protein Bax. Figure 5 shows a representative Western blot following drug treatment in Chang cells while Figure 6 summarizes the data for all studies. Diltiazem (2.5 µM and 10 µM) significantly reduced the levels of Bax from control cells (no drug treatment; p<0.001). There was no statistical difference between the two diltiazem concentrations. The reduction in Bax levels was much greater in the presence of Silymarin than control (p<0.001) and significantly greater than with diltiazem (p<0.01 at 10 µM and p<0.001 at 2.5 µM diltiazem). The combination of diltiazem
and silymarin further reduced Bax levels. The data show that the combination of diltiazem and silymarin provides further hepatoprotection than either diltiazem or silymarin alone.

**Bax mRNA**

To further elucidate the protective nature of drug treatment we investigated the effects of diltiazem and silymarin on Bax mRNA to understand if the protective function is related to the degradation of the anti-apoptotic markers or suppressed transcription. Figure 7 shows a representative scan of RT-PCR Bax mRNA. It is apparent that diltiazem suppresses Bax mRNA significantly more than the use of silymarin alone.

**Summary**

Intracellular free radical levels were assessed using DCF fluorescence following exposure of cells to an oxidative stress of 400 μM H₂O₂ for 20 min. Diltiazem significantly reduced the DCF fluorescence signal following 5 and 10 μM as did Silymarin at 1000 μg/L. The combination of diltiazem and silymarin further protected the cells against oxidative stress (p<0.001) compared to either drug alone. Cell viability was assessed using the MTT assay which showed that diltiazem and silymarin statistically enhanced cell growth (p<0.01) with the combination also providing a further protective effect. The nature of the protective effect was assessed by investigating levels of ATP, Bax and Bax mRNA. Diltiazem, silymarin and the combination drug treatment were all associated with enhanced ATP levels in both cell types with the combination drug treatment showing a much greater protective effect (p<0.001). Bax and Bax mRNA levels were statistically reduced by drug treatment, again with the combination showing a much greater beneficial effect than either treatment alone (p<0.001). We conclude that low dose diltiazem and silymarin (1 μg/ml) provides a hepatoprotective effect against free radical damage due to oxidative stress. The combination of diltiazem and silymarin provided an additional hepatoprotective
effect compared with either drug alone. The protective nature extends to reducing levels of Bax a pro-apoptotic protein.

**Example 2: Metabolism Studies of Diltiazem and Silymarin**

The procedures in this Example are conducted with a rat liver cell line, the H4-IIIE cell line. This is a well established model liver cell. The cell line exhibits all major characteristics of normal differentiated rat hepatocytes. The cell line has been employed in Medical Biochemistry research on liver function for the last 20 years and its properties compared favourably with those of normal freshly-isolated rat hepatocytes. Oxidative damage is induced using an established method which employs hydrogen peroxide. Liver cells in culture in plastic multiwall plates are incubated with 0.5 mM hydrogen peroxide for 1 hour, then for 12 hours in normal medium under standard incubation conditions. This regime induces oxidative damage to target cells. Cell damage is assessed by measuring exclusion of the dye Trypan blue and release of the cytoplasmic enzyme, lactate dehydrogenase.

To assess rates of silymarin metabolism by liver cells, the cells are incubated with a 5 μM concentration of silymarin for 4 - 10 hours under normal cell culture incubation conditions. The experiments are conducted with normal untreated liver cells and cells previously treated with hydrogen peroxide to induce oxidative damage. The metabolism of silymarin through the liver conjugation and oxidative drug metabolising enzyme pathways is measured using liquid chromatography in order to separate the different metabolites and mass spectrometry in order to identify and quantitate the metabolites.

To test the effects of diltiazem, cells are incubated in presence and absence of 5 μM diltiazem. The experiments are conducted with normal untreated cells and cells treated with hydrogen peroxide as described above. Each is incubated in the presence and absence of diltiazem. Silymarin metabolites will be measured using liquid chromatography and mass spectrometry as described. The degree
of damage of the cells induced by hydrogen peroxide is assessed using measurement of lactate dehydrogenase activity in the culture medium.

Cells exposed to oxygen free radicals have slowed metabolism of silimarín. The addition of low doses of diltiazem help maintain the ability of damaged cells to metabolise silimarín and related compounds.

This effect may be allied to the protection and enhancement of the mitochondrial function enabling preservation of energy production. It may be that this effect of diltiazem on the mitochondria enable liver cells to more effectively metabolise silymarin than would otherwise be the case when they are exposed to oxygen free radicals, or are mildly damaged.

This finding significantly reduces the risk of side effects from the build up of silymarin and its metabolites and provides a treatment with a constant low level of actives, a short half life and good first bypass clearance even in diseased liver cells for which silimarín and its metabolites may otherwise build up and result in side effects.
Claims

1. A method of treatment of a subject suffering from liver disease comprising the administration of (i) an oral slow-release formulation of a calcium channel blocker with antioxidant effects and (ii) at least one flavonolignan.

2. A method according to claim 1 wherein the calcium channel blocker and flavonolignan are administered in the same slow-release formulation.

3. A method according to any one of the previous claims wherein the flavonolignan is administered in a separate oral dosage form.

4. A method according to any one of the previous claims wherein thiamine is co-administered in addition to the calcium channel blocker and flavonolignan.

5. A method according to any one of the previous claims wherein the at least one flavonolignan is selected from the group consisting of Silibinin, silymarin, flavonolignans extracted from blessed milk thistle (Silybum marianum), pharmaceutically acceptable salts of silibinin such as silibinin dihydrogen disuccinate disodium, inclusion complexes of silibinin and glycosides of silibinin; and the calcium channel blocker is diltiazem.

6. A method according to any one of the previous claims wherein the daily dosage of flavonolignan is in the range of from 1 to 5000mg/day and the daily dosage of calcium channel blocker is from 10 to 70 mg / day of diltiazem.

7. A method according to claim 2 wherein the slow release formulation comprises a unit dose comprising the calcium channel blocker diltiazem in an amount from 20 to 70mg and the flavanolignan in an amount of from 10 to 2000 mg.
8. A method according to any one of the previous claims wherein the weight ratio of the daily dosage of diltiazem:flavonolignan is in the range of from 2:1 to 1:120.

9. A method according to any of the previous claims wherein the energy-preservation effect of diltiazem on the mitochondria enables damaged liver cells to continue to metabolize the flavonolignan thereby maintaining the short half-live of silymarin and the liver-selective delivery of the same.

10. A method according to any one of the previous claims wherein at least the calcium channel blocker is in a slow-release formulation providing a mechanism of release selected from the group consisting of polymer-coated multiparticulates, polymer-coated tablets or minitablets, or hydrophilic matrix tablets.

11. A method according to any one of the previous claims wherein the calcium channel blocker and flavonolignan are administered in at least one dosage in the form of a capsule, tablet or caplet.

12. Use of (i) a calcium channel blocker with antioxidant effects and (ii) at least one flavonolignan, which is also an antioxidant in preparation of a medicament for treatment of liver disease by coadministration thereof in one or more compositions for providing slow-release of the calcium channel blocker.

13. A pharmaceutical composition for treatment or prevention of liver disease comprising (i) an oral slow release formulation of a calcium channel blocker with antioxidant effects and (ii) at least one flavonolignan.
14. A composition according to a claim 13 wherein the at least one flavonolignan is selected from the group consisting of Silinin, silymarin, flavonolignans extracted from blessed milk thistle (Silybum marianum), pharmaceutically acceptable salts of silibinin such as silibinin dihydrogen disuccinate disodium, inclusion complexes of silibinin such as \( \beta \)-cyclodextrin inclusion complexes and glycosides of silibinin.

15. A composition according to claim 13 or claim 14 wherein the calcium channel blocker is diltiazem.

16. A composition according to any one of claims 13 to 15 wherein the composition is in a unit dosage form comprising from 20 to 70mg of calcium channel blocker.

17. A composition according to any one of claims 13 to 16 wherein the ratio of the weight ration of diltiazem:flavonolignan is in the range of from 50:1 to 1:120 and more preferably from 2:1 to 1:120.

18. A composition according to any one of claims 13 to 17 wherein the composition is in a unit dosage form comprising from 50 to 3000mg flavonolignan component.

19. A composition according to any one of claims 13 to 17 wherein at least the calcium channel blocker is in a slow-release formulation providing a mechanism of release selected from the group consisting of polymer-coated multiparticulates, polymer-coated tablets or minitablets, or hydrophilic matrix tablets.

20. A composition according to any one of claims 13 to 19 wherein the controlled release formulation of calcium channel blocker provides a dose delivery rate sufficient to provide a clinical effective blood level of calcium.
channel blocker in the portal vein and less than required to provide a clinically effective level in the peripheral circulation to thereby provide a delivery rate having a selective effect on the liver.

21. A composition according to any one of claims 13 to 20 wherein the calcium channel blocker and flavonolignan are together present in at least one unit dosage form selected from a capsule, tablet or caplet.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Bax Levels

Absorbance units

Drug Treatment

Figure 6
RT-PCR

- Bax
  - Control
  - Control H2O2
  - 2.5 μM Dithizone
  - 10 μM Dithizone
  - 10 μg/L Silvamar
  - 1000 μg/L Silvamar
  - 2.5 μM Dithizone + 1000 μg/L Silvamar
  - 10 μM Dithizone + 1000 μg/L Silvamar
  - 487 bp

- GAPDH
  - 349 bp

Figure 7
INTernational Search Report

A. CLASSIFICATION OF SUBJECT MATTER
   Int. Cl.
   A61K 36/28 (2006.01)  A61P 1/16 (2006.01)
   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)

   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)
   WPIDS, EPODOC, MEDLINE: (Keywords; Calcium Channel Blocker, Diltiazem, verapamil, amiodipine, niirendipine, silymarin and like terms)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<tr>
<th>Category (A)</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tr>
<td>X</td>
<td>OLIVERIA, C.P.M.S. et al. &quot;Protection Against Liver Ischemia-Reperfusion Injury in Rats by Silymarin or Verapamil. Transplantation Proceedings.&quot; 2001, vol.33, pages 3010-3014. See Materials and Methods, Study Groups and Table 1</td>
<td>1-21</td>
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Further documents are listed in the continuation of Box C [X] See patent family annex

Date of the actual completion of the International search: 03 November 2010

Date of mailing of the international search report: 12 Nov 2010

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Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX