Sphingolipids in essential hypertension and endothelial dysfunction
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FTY720 (Fingolimod) increases vascular tone and blood pressure in spontaneously hypertensive rats via inhibition of sphingosine kinase

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Summary

FTY720 (Fingolimod) is a recently approved orally administered drug for the treatment of multiple sclerosis. Phase II and III clinical trials have demonstrated that this drug modestly increases blood pressure (BP). We have previously shown that inhibition of sphingosine kinase increases vascular tone and BP in hypertensive, but not normotensive rats. Since FTY720 is reported to have sphingosine kinase inhibitory effects, we investigated whether FTY720 increases vascular tone and BP only in hypertensive rats via this mechanism. The contractile and BP modulating effects of FTY720 were studied in vivo and ex vivo (wire myography) in age-matched normotensive Wistar Kyoto (WKY) rats and spontaneously hypertensive rats (SHR).

Oral administration of FTY720 induced an increase in mean arterial pressure in SHR, whereas a decrease in BP was observed in WKY rats, as measured 24 hours after administration. In analogy to the sphingosine kinase inhibitor dimethylsphingosine (DMS), FTY720 induced major contractions in isolated carotid arteries from SHR, but not in those from WKY. In contrast, the phosphorylated form of FTY720 (FTY720-P) did not induce contractions in isolated carotid arteries from SHR. FTY720-induced contractions (like DMS-induced contractions) proved to be endothelium-dependent and to be mediated by thromboxane A2, since these contractions could be inhibited by endothelium denudation, cyclooxygenase and thromboxane synthase inhibitors and by thromboxane receptor antagonism. In conclusion, these data demonstrate that FTY720 increases vascular tone and BP only in hypertensive rats, most likely due to its sphingosine kinase inhibitory effect.
Introduction

The immunosuppressant drug FTY720 (Fingolimod; 2-amino-[2-(4-n-octylphenyl)ethyl]-1,3-propanediol) is a recently approved therapeutic addition to the treatment options of relapsing multiple sclerosis\(^1\). FTY720 is a sphingosine analogue that is phosphorylated \textit{in vivo} to (S)-FTY720-P by sphingosine kinase (mainly type 2)\(^2,3\), the enzyme responsible for the production of the endogenous bioactive sphingolipid sphingosine-1-phosphate (S1P). (S)-FTY720-P is a high affinity ligand at four of the five S1P-receptors (S1P\(_{1,3,4,5}\)) and leads to degradation of S1P\(_1\) receptors on T-lymphocytes, that subsequently results in a reduced lymphocyte egress and thus a T-lymphocyte specific immunosuppression\(^4,6\). In phase II and III clinical studies, a modest rise in blood pressure (BP) (3-5 mmHg) was detected in patients treated with FTY720\(^7,9\). Although the active metabolite (S)-FTY720-P has vasoactive properties (both vasodilation and vasoconstriction have been reported)\(^10,11\), until now the exact mechanism by which FTY720 induces this rise in BP \textit{in vivo} remains elusive.

We have previously shown that hypertension, both experimental as well as human essential hypertension, is associated with profound alterations in vascular sphingolipid biology\(^12\). Sphingolipids, such as ceramide and S1P, are bioactive lipids that play an important role in cellular signaling. They do not only play a crucial role in cell growth\(^13,14\), but they are also involved in vascular function\(^15-18\). S1P for instance, via activation of endothelial S1P receptors, is known to activate eNOS and thereby to induce vasodilation\(^19,20\). In contrast, we have shown that ceramide, a precursor of S1P, potently stimulates endothelium-mediated TXA\(_2\) synthesis in isolated carotid arteries of spontaneously hypertensive rats, thereby inducing endothelium-dependent vasoconstriction\(^12\). Interestingly, the latter phenomenon is only present in vessels from hypertensive rats, because of an increased endothelial expression of the enzymes involved in TXA\(_2\) synthesis. Via this mechanism also pharmacological inhibition of sphingosine kinase by means of dimethylsphingosine (DMS) induces major endothelium-dependent vasoconstrictions in isolated carotid arteries from SHR, but not in those from normotensive rats. Moreover, inhibition of sphingosine kinase in SHR \textit{in vivo} results in a marked rise of BP, while it has no effect, or even lowers BP in normotensive WKY rats\(^12\).

While FTY720 is phosphorylated mainly by sphingosine kinase type 2, it has been reported to be a potent inhibitor of sphingosine kinase type 1\(^21,22\). Therefore, we hypothesized that FTY720, in analogy to DMS, increases vascular tone and BP in hypertensive but not
normotensive rats. Here we show that FTY720 indeed elevates BP solely in hypertensive rats and induces vasoconstriction in isolated carotid arteries from SHR via thromboxane A₂ production.

**Methods**

**Chemicals**
Acetyl-β-methylcholine (methacholine), phenylephrine, indomethacin and ozagrel were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Dimethylsphingosine (DMS) from Biomol International L.P. (Plymouth, PA, USA), and SQ29548 from Alexis Biochemical (San Diego, CA, USA). FTY720 and FTY720-P were synthesized according to previously described methods ³. All other chemicals were from Sigma Aldrich and of analytical grade.

**Animals**
Six-months-old male spontaneously hypertensive rats (SHR) and Wistar Kyoto rats (WKY) were purchased from Charles River (Maastricht, The Netherlands) and handled accordingly to a protocol approved by the Animal Ethical Committee of the University of Amsterdam, the Netherlands. The animals were housed with access to food and water ad libitum under a 12h light/dark cycle. For myography experiments, rats were anaesthetized by i.p. injection of 75 mg/kg pentobarbital (O.B.G., Utrecht, The Netherlands). Heparin (750 IU, Leo Pharma B.V., Weesp, The Netherlands) was injected intraperitoneally to prevent blood coagulation and thrombocyte-derived S1P release.

**Tail-cuff blood pressure measurements**
FTY720 (0.3 mg/kg) was given orally by gastric gavage. For awake BP measurements, 24h after FTY720 administration, the CODA™ monitor (Kent Scientific Corporation, CT, USA) was used. In brief, rats were fixed in a transparant animal holder and placed on a heating pad. The rat was left untouched and fixated for a couple of minutes before placing the tail-cuffs. Then, tail-cuffs were placed loosely fitting over the tail slightly below the tail base. An average of 8 repeated tail-cuff cycles were performed per rat per condition. During the experiment, care was taken to ensure minimal stress development in the animals.
Immunohistochemistry and quantification

Carotid artery segments from untreated SHR and WKY rats were collected directly after dissection and rapidly submerged in OCT Compound (Sakura, TissueTek) and frozen in liquid nitrogen with subsequent storage at -80°C. Frozen sections (5 µm) were cut on a Leica CM3050S cryostat and dried by cold pressurized air and fixed in 100% acetone during 1 min.

Then, slides were washed shortly in 0.1% PBS/BSA (w/v) and incubated with blocking buffer (2% PBS/BSA) during 30 min at room temperature. After a short wash, slides were incubated with the primary antibody against sphingosine kinase 1 (Cayman Chemical Co., Ann Arbor, MI, USA, #10006822; 1/50 dilution) dissolved in 0.1% PBS/BSA overnight at 4°C.

Following a triple wash in 0.1% PBS/BSA during 5 min, the appropriate A546-labelled secondary antibody (Invitrogen, Carlsbad, CA, USA, #A-11010; 1/400 dilution) was applied during 1 hour at room temperature. After triple wash, the antibody against von Willebrand Factor (GeneTex, Irvin, CA, USA, #GTX74830; 1/200 dilution) was applied during 1 hr at room temperature as marker of the endothelium. After triple wash, the final A488-labelled secondary fluorescent antibody (Invitrogen, #A-11029, 1/400 dilution) was applied. Finally after triple wash, DAPI containing mounting medium (Santa Cruz Biotechnology (Santa Cruz, CA, USA, #sc-24941) was applied and vessels were imaged using a Nikon Eclipse TE2000-U fluorescence microscope (Plan Fluor ELWD 20x objective, Nikon DXM1200F digital camera) with NIS Elements AR 2.30 software. The region of interest was determined of each segment by detection of the endothelial marker von Willebrand Factor, without any information on the protein to quantify to ensure unbiased recording. Then the appropriate filter setting was chosen to record the mean fluorescence intensity using the NIS Elements software on the raw unprocessed images. For both endothelium and smooth muscle cell determinations, an intensity threshold was selected to exclude background fluorescence. All settings and exposure times were applied to all slides equally for the appropriate protein to quantify.

Arterial preparation and isometric force recording

The left common carotid artery was carefully excised in a range just distal from the bifurcation until the level of the aortic arch and immediately placed in Krebs-Henseleit buffer (pH7.4; in mmol/L: 118.0 NaCl, 4.7 KCl, 25.0 NaHCO₃, 1.2 MgSO₄, 1.8 CaCl₂, 1.1 KH₂PO₄ and 5.6 glucose) at room temperature, aerated with 5% CO₂ / 95% O₂, pH 7.4. Four segments of carotid artery were carefully prepared and two stainless steel wires with a diameter of 40 µm (Goodfellow, Huntingdon, U.K.) were inserted into the lumen of each vessel segment. In selected cases the endothelium was removed mechanically (by rolling a luminaly-inserted PE-50
tube several times) before mounting. The segments were then transferred into organ baths of a
4-channel wire myograph (610M, Danish Myo Technology, Aarhus, Denmark) and subjected to
a normalization procedure according to Mulvany & Halpern. In short, the individual
circumference was adjusted to 90% of the value that the particular vessel would have had at a
transmural pressure of 100 mmHg. Afterwards, the arteries were equilibrated for 30 min and
the buffer was refreshed after each period of 15 min. The preparations were contracted twice
for 10 min with a depolarizing high K+ Krebs-Henseleit solution (100 mmol/L NaCl was replaced
by 100 mmol/L KCl) at intervals of 15 min. Subsequently, the vessels were pre-contracted with
the α1-adrenoceptor agonist phenylephrine (0.3 µmol/L). After reaching a steady level of a
contraction force >60% of previous KCl-induced depolarization contraction, one concentration
(10 µmol/L) of the endothelium-dependent vasodilator methacholine was added to assess the
endothelial integrity. For endothelium-denuded vessels, a relaxation <5 % indicated successful
denudation, and these segments were included accordingly. After washing, again 100 mmol/L
KCl was added to the vessel segments. After washing and a 30 min pre-incubation of inhibitors
or their vehicle (in all cases DMSO), DMS (10 µmol/L), FTY720 (10 µmol/L) or FTY720-P (10
µmol/L) were added and the vascular responses were recorded for an additional 50 min.
Isometric force of contraction was measured continuously and all data are presented in mN/mm
segment length (except for raw tracings).

Data analysis and statistics
The isometric tension measurements in carotid artery segments and BP/heart rate
measurements are presented as mean ±SEM with ‘n’ being the number of individual rats. Peak
contraction values during the myography experiments were determined and expressed as
relative tension (mN/mm) and presented in column graphs. Statistics were performed by
student’s t-test (Fig. 2-4) and one-way ANOVA including Dunnett’s multiple comparisons test
(95% confidence interval) with FTY720 values as control (Fig. 5). All statistical analyses were
performed using Prism (GraphPad Prism Software, San Diego, CA, USA). Values of p<0.05 were
considered to be statistically significant.

Results

Oral challenge with FTY720 elevates blood pressure in vivo in SHR, but not WKY
Figure 1 shows the structural similarities of FTY720 and the sphingosine kinase inhibitor DMS,
both derivatives of sphingosine, the natural substrate for sphingosine kinases.
To investigate whether FTY720, like DMS, raises BP preferentially in hypertensive animals, SHR and WKY rats were given oral FTY720 (once 0.3 mg/kg in saline) and BP/heart rate were recorded using tail-cuff non-invasive BP measurements before and 24 hours after FTY720 challenge. Mean arterial pressure (MAP) of untreated WKY rats before was 125.0±3.4 mmHg and SHR 157.5±2.3 mmHg (n=4-7, p<0.05). 24h after the oral dose of FTY720, WKY BP was lowered to 109.5±9.7 mmHg, whereas BP in SHR was elevated to 170.6±5.2 mmHg (n=4-7, p<0.05)(Fig. 2A). Heart rate, however, was equally reduced in WKY and SHR (WKY: 356±7 before vs 331±9 bpm after FTY720 and SHR: 433±10 before vs 403±23 bpm after FTY720, n=3-7, p>0.05) (Fig. 2B).

Figure 2. Blood pressure response to oral FTY720 challenge in SHR and WKY rats. A) Tail-cuff mean arterial pressure (MAP) measurement in WKY and SHR before (0h) and after 24 hours (24h) of oral (0.3 mg/kg FTY720) challenge and B) corresponding heart rate (HR). Data expressed as mean ±SEM, n=4, * p<0.05.
Sphingosine kinase 1 expression is elevated in SHR carotid artery segments compared to those of WKY

Since the main target of DMS and unphosphorylated FTY720 is sphingosine kinase, the protein expression profile of sphingosine kinase 1 in carotid arteries of WKY and SHR was assessed. As indicated by immunohistochemistry, in both the endothelium and smooth muscle layer of isolated SHR carotid artery segments, sphingosine kinase 1 was higher expressed compared to WKY carotid artery segments (Fig. 3).

DMS and FTY720 induce profound contractile responses in isolated carotid artery segments of SHR but not in those of normotensive WKY rats

While completely unresponsive in isolated carotid arteries of normotensive WKY rats, DMS (10 µmol/L) induced profound transient contractions in artery segments of spontaneously hypertensive rats as described previously 12 (a DMS tracing is shown as a reference in Fig. 4A and 4B). In analogy to DMS, FTY720 did not evoke any response in carotid artery segments from WKY rats (Fig. 4C and 4F), but induced major transient contractions in segments from SHR (Fig. 4D and 4F). Importantly, in contrast to its parent compound, the phosphorylated form of FTY720 (i.e. FTY720-P) did not induce any substantial response in segments from SHR. Thus

Figure 3. Quantitative immunohistochemistry on sphingosine kinase 1 expression. A) Typical example of WKY and SHR carotid artery expression profile of sphingosine kinase 1 (red) and cell nuclei (blue; left) and additionally with vWF endothelium marker (green; right). B) staining intensity quantified as endothelium (EC)-specific expression and vascular smooth muscle cell (VSMC)-specific expression. Data expressed as mean fluorescent light units (FLU) ±SEM, n=5-6, * p<0.05.
only unphosphorylated FTY720 induced transient vasoconstrictions, only in carotid arteries of SHR.

Figure 4. Contractile effect of DMS, FTY720 and FTY720-P on carotid arteries of SHR and WKY.
Typical tracings showing the contractile effects of DMS (A + B), FTY720 (FTY, C + D), and FTY720-P (FTY-P, E) in isolated carotid arteries from WKY rats (A +C) and SHR (B, D + E). Please note the profound endothelial dysfunction in arteries from SHR as evidenced by a decreased relaxant response to methacholine (MCh; 10 µmol/L) in phenylephrine (Phe; 0.3 µmol/L) pre-contracted artery segments. Quantified data of FTY720-induced effects (F) are expressed as mean ± SEM in mN/mm segment length, n=3-6, * p<0.05.

and not in those of WKY (FTY720 in WKY 0.1±0.0, FTY720 in SHR 1.8±0.3, FTY720-P in WKY 0.1±0.0, FTY720-P in SHR 0.1±0.1 mN/mm, n=3-6; p<0.05) (Fig. 4F).

FTY720 and DMS induce vasoconstriction in carotid artery segments of SHR via a similar mechanism
DMS-induced vasoconstriction has previously been shown to be endothelium-dependent and to be sensitive to cyclooxygenase and thromboxane A₂ synthase inhibition and could be antagonized by the thromboxane/prostaglandin (TP) receptor antagonist SQ29548. To investigate whether FTY720 and DMS induce contractions in carotid artery segments from SHR via a similar mechanism, we either removed the endothelium or pre-incubated the vascular
segments with DMS, applied inhibitors of aforementioned enzymes or the TP-receptor antagonist prior the addition of FTY720. FTY720-induced vasoconstriction was diminished in DMS-pretreated segments (FTY720 was added directly after the initial DMS-induced constriction has relaxed) (0.57±0.19 mN/mm, n=4, p<0.05). Also endothelial denudation blunted the contractile response to FTY720 (0.2±0.2 mN/mm), indicating that the contractions were indeed endothelium-dependent (Fig. 5). Furthermore, the cyclooxygenase inhibitor indomethacin (10 µmol/L), the thromboxane synthase inhibitor ozagrel (10 µmol/L) and the TP receptor antagonist SQ29548 (10 µmol/L) were all able to inhibit FTY720-induced vasoconstriction (0.3±0.3, 0.1±0.1 and 0.1±0.1 mN/mm respectively, n=4-6, p<0.05) (Fig. 5).

Figure 5. Mechanism of FTY720-induced contractions in isolated carotid arteries from SHR. FTY720-induced contractions in the absence of endothelium (-EC), in the presence of the cyclooxygenase inhibitor indomethacin (10 µmol/L), the thromboxane synthase inhibitor ozagrel (10 µmol/L) and the TP receptor antagonist SQ29548 (10 µmol/L). Data expressed as mean ±SEM in mN/mm segment length, n=4-6, * p<0.05.
Discussion

FTY720 (Fingolimod) is a recently approved oral treatment option for multiple sclerosis. In clinical trials, FTY720 as compared with placebo, had a superior efficacy over a 2-year period in patients with relapsing–remitting multiple sclerosis, and proved more effective than intramuscular interferon beta-1a over a 12-month period. Phase II and the aforementioned phase III clinical trials have shown that FTY720 is generally well tolerated and the reported adverse effects are, next to adverse effects due to immunosuppression, also of cardiovascular nature. Besides transient effects on heart rate (bradycardia) and atrio-ventricular conduction, a moderate increase in BP of approximately 3-5 mmHg in 4 to 6% of treated patients was observed that persisted during treatment. In addition, one case of severe peripheral arterial vasospasm was reported in a patient after 7 days of FTY720 treatment.

In a previous report we have shown that hypertension is associated with marked alterations in vascular sphingolipid biology. We have shown that shifting the ceramide/S1P ratio towards ceramide, for instance by pharmacological inhibition of sphingosine kinase, can trigger endothelium-dependent production of thromboxane A2 in hypertensive animals, thus inducing vasoconstriction. Accordingly, intravenous infusion of DMS induces a marked increase in BP in anesthetized SHR, whereas it has no effect, or even lowers BP in WKY rats. Interestingly, ceramide levels in arterial tissue and plasma were significantly higher in SHR compared to those in normotensive WKY rats. The fact that also humans with stage 2/3 hypertension display elevated levels of ceramide in blood plasma, indicates that also human essential hypertension is associated with alterations in sphingolipid biology. Since several reports have clearly demonstrated that FTY720 has profound inhibitory effects on sphingosine kinase we were prompted to investigate whether FTY720, like the sphingosine kinase inhibitor DMS, increases vascular tone and BP only in SHR.

In order to investigate whether FTY720 shows a similar divergent behavior as DMS in our previous study, we applied FTY720 in vivo. Intravenous infusion of FTY720 in anesthetized rats, however, caused marked effects on cardiac frequency, making it rather difficult to measure BP responses. As mentioned before, the cardiac effects of FTY720 are well known and have been described in laboratory animals and humans. Since the bradycardia induced by FTY720 is known to be transient, we decided to administer FTY720 orally and measure the BP response after 24 hours. While in WKY rats orally administered FTY720 reduced BP, we observed an increase in BP in SHR as measured 24h after application. At this time point heart rate was still...
reduced in both groups to the same extend. The latter excludes the possibility that the changes in heart rate are responsible for the divergent response in BP we observed. These experiments confirm that FTY720 induces comparable BP responses in normotensive and hypertensive animals as the sphingosine kinase inhibitor DMS.

In order to investigate whether similar mechanisms are involved, we performed ex vivo experiments in isolated carotid arteries. In these blood vessels, sphingosine kinase 1 expression is elevated in SHR compared to WKY, suggesting aggravated sensitivity to both DMS and FTY720 inhibition. Myography experiments clearly demonstrate that FTY720, like DMS, induces vasoconstriction only in arteries from hypertensive rats and not in those from normotensive WKY rats. In contrast, FTY720-P, the phosphorylated derivative of FTY720 does not induce constriction in artery segments of neither WKY nor SHR. This is most likely due to the fact that FTY720-P has no sphingosine kinase inhibitory effects. This finding excludes the possibility that the constriction to FTY720 is caused by S1P receptor stimulation via FTY720-P, that will be formed via sphingosine kinase 2 activity in the artery segment.

The induction of a transient vasoconstriction of FTY720 in isolated carotid arteries from SHR but not WKY rats closely resembles the vascular effects of DMS. In addition, also the fact that FTY720-induced contractions are substantially diminished in segments pre-treated with DMS, suggests that DMS and FTY720 induce vasoconstriction via a similar mechanism. The DMS-induced contractions, proved to be endothelium-dependent and to be mediated via the eicosanoid thromboxane A₂ 12. Indeed, also FTY720-induced contractions were abolished by mechanical removal of the endothelium prior the addition of the compound. Moreover, the fact that the contractions were potently inhibited by cyclooxygenase and thromboxane synthase inhibition and to TP-receptor antagonism, indicates that FTY720 and DMS induce constriction via a similar mechanism. In our previous report we demonstrated that increased expression of calcium-independent phospholipase A₂, cyclooxygenase and thromboxane synthase (all enzymes involved in TXA₂ synthesis) in carotid artery segments of SHR, accounts for this phenomenon.

Whether this mechanism (i.e. sphingosine kinase inhibition) also contributes to the BP increasing effect of FTY720 in humans remains elusive. Although these data are unfortunately not available in literature, it would be interesting to see whether the observed increases in BP in multiple sclerosis patients are specifically in those patients that already had hypertension or endothelial dysfunction.

Besides the sphingosine kinase inhibitory effect of the unphosphorylated FTY720, after phosphorylation to FTY720-P in vivo, it may induce vasoconstriction via stimulation of S1P
receptors on smooth muscle cells. However, vasoconstriction to FTY720-P is restricted to particular vascular beds, such as coronary and basilar arteries. As we show in this current study, FTY720-P does not induce vasoconstriction in carotid arteries. In other, peripheral, arteries FTY720-P induces an endothelium-dependent vasodilation via stimulation of endothelial S1P₁ and/or S1P₃ receptors. Therefore, it is unlikely that stimulation of vascular S1P receptors exclusively contributes to the BP increases as observed in patients treated with FTY720.

In addition, this is not explanatory for the divergent responses to FTY720 in normotensive and hypertensive animals. In this regard it is important to realize that the carotid artery is a conduit and not a resistance vessel. Interestingly, we do not observe contractile responses to FTY720 and DMS in mesenteric arteries from SHR or WKY; in contrast, we do observe vasodilation to FTY720 in pre-constricted mesenteric arteries (data not shown). This may be in accordance with observations that sphingosine kinase 1 expression in mesenteric arteries is rather low compared to cerebral vessels. The fact that we observe clear BP increases to FTY720 in SHR, however, implicates that certain resistance vessels in vivo do contract. This may be due to direct TXA₂ release in vivo in the resistance vascular bed itself, or to paracrine/endocrine effects of TXA₂ released from other vascular beds.

Another important aspect to keep in mind when extrapolating the in vitro data to the in vivo situation is metabolization of FTY720. One could argue that FTY720 in vivo is rapidly converted to FTY720-P, and that in contrast to the in vitro situation the vasculature in vivo is mainly exposed to FTY720-P. However, several pharmacokinetic studies have demonstrated that FTY720 is absorbed very slowly; peak FTY720 plasma levels in are reached approximately 36 hours after oral administration of a single dose. A recent study performed with radiolabelled FTY720 in humans clearly demonstrates that FTY720 is only partially phosphorylated in vivo. Because of similar half-life values of FTY720 and FTY720-P, the ratio between the pro-drug and the metabolite remains rather constant and amounts to approximately 2. Thus also in vivo, endothelial cells are exposed to higher concentrations of FTY720 than FTY720-P.

Next to inhibition of sphingosine kinase, FTY720 has been reported to inhibit cytoplasmic PLA₂ and ceramide synthase. It is unlikely, however, that these properties are involved in the contractile effects of FTY720 as reported here; because of the role of arachidonic acid metabolites and ceramide in vessels from SHR as mentioned before, one would expect the opposite effect.
In conclusion, we clearly demonstrate that FTY720 induces vasoconstriction in isolated carotid arteries and raises BP in hypertensive, but not normotensive animals, most likely via its inhibitory effect on sphingosine kinase. Whether this mechanism contributes to increases in BP during FTY720 treatment in humans, remains to be investigated.
References


Chapter 5


