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► **To cite this version:**

Clifford W Fong. Statins in therapy: Cellular transport, side effects, drug-drug interactions and cytotoxicity -the unrecognized role of lactones. [Research Report] Eigenenergy, Adelaide, Australia. 2016. hal-01185910v2

HAL Id: hal-01185910

<https://hal.science/hal-01185910v2>

Submitted on 2 Jul 2016

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Statins in therapy: Cellular transport, side effects, drug-drug interactions and cytotoxicity - the unrecognized role of lactones

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Keywords: Statins, statin lactones, cytotoxicity, multivariate models, molecular orbital computations, membrane transport

Abbreviations: drug-drug interactions (DDI), human serum albumin (HSA), area under the curve fold ratio (AUCR), free energy change (ΔG), electron affinity (EA), reactive oxygen species (ROS), electrochemical peak reduction potentials (E_p), linear multivariate correlation coefficient (R), standard error of the estimate (SEE), standard error (SE), anova F test statistic (F)

Abstract

A molecular orbital computational study of the statins and a comprehensive review of the literature have found that the lactone forms of statins play a major role in the transport of statins across the cell membrane, and in the metabolism and clearance from the body. The neutral lactone and acid statin species are preferentially transported across the cell membrane and consequentially preferentially metabolised and cleared. The preferred cellular uptake of statin lactones has implications for cytotoxicity in muscle tissue and other side effects. The uptake mechanism is a combination of passive facilitated diffusion and active permeation by OATP transporters. Quantitative models describing how uptakes rates, binding affinity between statins and OATP transporters, and the inhibition of statin-OATP transporters processes are related to the four principal determinants of cell membrane permeability (desolvation, lipophilicity, dipole moment and molecular volume) have been deduced. Cyclosporin and gemfibrozil competitive inhibition of statin-CYP metabolism correlates with desolvation, lipophilicity, dipole moment and electron affinity, which suggests that electron affinity, a measure of reduction potential, is a useful indicator of potential drug-drug interactions (DDI). A similar relationship was found with the statin inhibition of paclitaxel oxidation by CYP enzymes. An examination of drugs known to cause DDI with statins show that reduction potential and cellular uptake properties are useful predictors of DDI. These statistical models, supported by literature evidence, indicate the statin lactones play a previously unrecognized major role in statin therapeutics, and in side effects, cytotoxicity and DDI. It is likely that protonated statin lactones are the active species involved in electron transfer from the mitochondrial electron transfer processes that are a major cause of statin lactone induced myopathy. It has been demonstrated that the four parameter equation incorporating the independent variables desolvation, lipophilicity, dipole moment and molecular volume (or electron affinity) successfully correlates with passive and active transport processes, the statin inhibition of CYP metabolic enzymes, and the inhibition of HMGCoA reductase by statin anions.

Objectives:

- To understand the roles that statin acids and lactones play in statin therapeutics
- To investigate the side effects of statin therapeutics by understanding the transport of statins across cell membranes
- To investigate the metabolism and clearance of statins from the body
- To investigate the cytotoxicity and drug-drug interactions of statins

- To develop quantitative mechanistic models for statin inhibition, transport and metabolic processes

1. Introduction

Statins are among the most widely prescribed drugs in the world. They are effective in lowering blood cholesterol levels, high levels of which can cause arteriosclerosis, and be detrimental to cardiovascular health. Statins can lower the risk of angina, heart attack and strokes. There can be other benefits other than cholesterol lowering effects, such as reducing the risk of esophageal cancer. While statins taken alone have shown remarkably few side effects, it is clear that patients taking multiple drugs for other medical conditions can experience side effects and even toxic effects when there are drug-drug interactions (DDI). [1] Statins are usually prescribed for people who have: atheroma-related diseases such as heart disease and arteriosclerosis by reducing the chance that these conditions will worsen and can delay progression of the diseases, diabetes or another disease that increases the risk of developing an atheroma related disease, those with a family history of heart attacks, and the elderly.

Common side effects are headache, pins and needles, abdominal pain, bloating, diarrhoea, feeling sick, rashes and rarely, muscle inflammation. Statins may raise the risk of cataracts. The rare major side effects are liver failure (increased levels of liver enzymes), slightly increased risk of diabetes, and skeletal muscle damage, rhabdomyolysis, which can occur when statins are used in combination with other drugs that carry high rhabdomyolysis risk, or with other drugs that unintentionally raise the statin levels in the blood. People with liver disease, pregnant and breast feeding women, are not advised to take statins. Statins should not be combined with medications such as protease inhibitors (AIDS treatment), clarithromycin, erythromycin, itraconazole, clarithromycin, diltiazem, verapamil, grapefruit juice, niacin, or fibrate drugs (that also lower cholesterol LDL levels). Statins have not been found to increase the risk of cancer, and may actually reduce the risk of esophageal cancer, colorectal cancer, gastric cancer, hepatocellular carcinoma, and possibly prostate cancer. There is some evidence that the use of coenzyme Q10 supplements may help to prevent statin side effects in some people. [2]

The efficacy of drug therapeutics is governed by several critical factors, including:

- The administered dosage, when and how much, and what form of the drug reaches its target: statins are administered orally, and can be in acid, anionic-salt, or lactone form. The pK_a , binding to blood serum or human serum albumin (HSA), method of transport across the cell membrane, both uptake and efflux, and drug lipophilicity are commonly considered critical factors.
- How the drug is metabolised or cleared from the body. Bioavailability, half life, volume of distribution are key systemic factors, and metabolism, usually by the cytochrome P450 (CYP) enzyme family, can clear the parent drug, or can form metabolites which can also be active drugs.
- The potency of the statins is related to all of the above factors, as well as the actual binding energy of the statins to the HMG-CoA enzyme, which inhibits cholesterol formation.
- A summary of these factors is given in Table 1.

Clinicians categorize potential DDI with statins particularly by those that are metabolized by the hepatic isoenzyme CYP3A4, such as simvastatin and to a lesser extent atorvastatin, which

are in combination contraindicated with drugs that *increase* systemic plasma levels of statins: (a) drugs that are potent inhibitors of CYP3A4 such as the macrolide antibiotics (clarithromycin, erythromycin), azole antifungals (itraconazole, ketoconazole), protease inhibitors (ritonavir), fibrates, cyclosporin etc; (b) other drugs that are moderate and lower inhibitors of CYP3A4, (c) drugs that induce CYP3A4 expression, and *reduce* statin bioavailability. Fluvastatin, pravastatin and rosuvastatin are not significantly metabolised by CYP3A4 and are clinically considered to be less susceptible to CYP interactions. [1,3] Pravastatin, rosuvastatin, and pitavastatin are excreted mainly unchanged, and their plasma concentrations are not significantly increased by pure CYP3A4 inhibitors.

The role of uptake and efflux cell membrane transporters has recently been recognized as important in metabolic clearance of statins, particularly the inhibition of these transporters by certain drugs. For example, cyclosporin inhibits CYP3A4, P-glycoprotein (multidrug resistance protein 1 or MDRP1), organic anion transporting polypeptide 1B1 (OATP1B1), and some other hepatic uptake transporters. Gemfibrozil and its glucuronide inhibit CYP2C8 and OATP1B1. Many drugs are now known to inhibit OATP1B1 and related transporters. [4-6]

While metabolism may be a critical factor in statin DDI, it is clear that there are other factors that affect systemic clearance rates and bioavailability, particularly trans-membrane transport processes and the actual statins species that are involved in the plasma, cytosol, metabolic oxidation reactions etc. *This study will focus on the relevance of the anionic, acid and lactone form of the statins and their relative importance in transport and metabolic processes.*

2. Discussion

2.1 Acid and lactone forms of statins:

Statins exist in both acid and lactone forms in vivo. Statins can be administered as the acid form, the anionic-salt form, or for lovastatin and simvastatin, often as the lactone form. The lactones are converted in vivo by carboxyesterases in the intestinal wall, the liver and partially in plasma, a fully reversible reaction. It is also known that glucuronidation can convert the open acid form of the statins, whether administered as the acid or lactone, to the lactone form eventually, which is about 10% of the administered dose of statin. [7] The lactones have lower aqueous solubility than the acid forms, and are not necessarily better absorbed. A study of atorvastatin acid and lactone, and the metabolized products, 2- and 4-hydroxyatorvastatin, found that the acid compounds were stable in human serum at room temperature but the lactone compounds were unstable as they hydrolysed rapidly to their respective acid forms. The lactone compounds in serum could be stabilized by lowering the temperature to 4° C or lowering the serum pH to 6.0. Conversion of the acid form to lactones is H⁺ catalysed to drive the acid \rightleftharpoons lactone equilibrium towards the lactone side. [8] Atorvastatin undergoes pH-dependent hydroxy acid–lactone interconversion similar to other statins. Under both mildly acidic and basic conditions, the lactone form is less stable than its hydroxy acid form, but in the presence of a carboxylic acid, the equilibrium is slightly shifted towards the lactone side (4 kcal mol⁻¹ difference). [9] The extracellular pH is usually about 0.5-0.6 higher than the intracellular pH, [10] a factor which also favours the lactone form in the cytosol.

Both lactone and acid forms were observed in the systemic circulation after oral administration of rosuvastatin [11] atorvastatin [12] simvastatin [13] lovastatin [7,15] and

cerivastatin [16] in humans and/or animals, indicating that interconversion occurs between the lactone and acid forms of these statins. When atorvastatin was administered in the acid form to patients, the lactone form was found in almost equal quantities.[12] The acid and lactone statins are also metabolized by the CYP enzymes, except pravastatin and rosuvastatin, with the lactones having a higher metabolic rate. [17,18] While it has been found that rosuvastatin is not significantly metabolized by the CYP enzymes, and is largely excreted unchanged, this situation is complicated by the finding that glucuronidation products of simvastatin acid, cerivastatin acid and rosuvastatin acid all undergo spontaneous cyclization to the lactone form.[7] Other statins (pitastatin, atorvastatin, fluvastatin) also undergo the glucuronidation conversion to lactones. [17,18] These observations indicate that statins that cross the cell membrane, either in the acid or lactone form, and could be interconverted in the cytosol, and also could undergo glucuronidation in the mitochondria to the acyl conjugates, which can then spontaneously cyclise to the lactone form.

2.2 Transport of statins across the cellular membrane

Hepatocellular transport is a key facilitator of metabolism and clearance of drugs. Statin metabolism and elimination is largely dependent on how well the liver can uptake, metabolize and clear these drugs. OATP1B1 is almost exclusively expressed in hepatocytes where it is involved in the uptake of bile acids, eicosanoids, DHEA, estrogens etc. OATP1B1 is known to transport all statins in current use. A comparison of all statins showed that simvastatin pharmacokinetics were strongly influenced by SLCO1B1 521C>T (3.2fold increase in AUC) followed by atorvastatin, then either pravastatin or rosuvastatin. Fluvastatin AUC was not affected by SLCO1B1 521C>T because it is influxed into the liver by other transporters. Individuals with SLCO1B1 polymorphism have reduced rate of statin uptake resulting in slower metabolism and elimination. [19]

Studies of hepatocellular transport have shown that it is a competitive blend of passive non-saturable diffusion and active saturable transport using transporters. Passive diffusion is almost certainly a facilitated diffusion process in view of the large size of the statins where concentration gradients down a trans-membrane pores are involved. [20]

2.3 Active transporters of statins:

The human OATP1B1-mediated transport of simvastatin acid, atorvastatin, pravastatin, pitavastatin, fluvastatin, rosuvastatin showed apparent K_m values between 0.6 to 29 μM , with atorvastatin having the highest and pravastatin the lowest affinity for OATP1B1. Simvastatin acid could not be determined due to its high passive permeability. [21] This data is consistent with the K_m values in Table 1 which indicates that the approximate binding energies of the statins with hepatocyte OATP transporters lies in the range -9.3 to -6.3 kcal/mol.

Corning TransportoCells OATP1B1 cells were used to characterize the intrinsic clearance rates for a series of statins, which showed a high correlation with the apparent hepatic uptake clearance rates in human hepatocytes. Pitastatin, pravastatin, rosuvastatin, simvastatin, atorvastatin and fluvastatin showed K_m values of 0.2, 95.4, 14.2, 0.8, 0.2, 0.95 μM with corresponding hepatic clearance of 232.9, 11.5, 2.6, 37.9, 193.5, 85.7 L/mg/min. Contributions of OATP1B1 to the total hepatic uptake were 0.93, 0.82, 0.77, 0.69, 0.47, 0.18 respectively. The passive permeation rates (in control cells) for the statins were 20.7, 0.6, 0.2, 13.8 (simvastatin acid), 6.3, 31.4, plus cerivastatin 88.4 and lovastatin acid 50 $\mu\text{L}/\text{mg}/\text{min}$.

[22] The passive permeation rates in the Corning TransportoCells correlated well with uptakes in human and rat hepatocytes. [23]

Atorvastatin uptake parameters for the OATP1B1, OATP1B3, OATP2B1 transporters were K_m 0.77, 0.73, 2.84 (μM) and V_{max} 6.61, 2.29, 24.27 (pmol/mg protein/min) respectively. Lovastatin acid, simvastatin acid and pravastatin acid were found to be specific OATP1B1 inhibitors. Pravastatin almost completely inhibits the uptake of atorvastatin by OATP1B1 (75%) with only a minor effect on OATP1B3 and OATP1B2 (15 and 5%) so overall atorvastatin uptake in the presence of pravastatin was reduced by 45%. [24]

The uptake of fluvastatin into rat cultured hepatocytes has been shown to be a combination of non-specific diffusion and specific partly ATP driven partly Na^+ dependent saturable active transport. The two enantiomers of fluvastatin showed specific concentration and temperature dependent uptake processes, with K_m and V_{max} for the (+) and (-) isomers of 38.5 and 41.5 μM and 611 and 646 $\text{pmol}(\text{mg protein})^{-1}\text{min}^{-1}$ respectively. Simvastatin also shows a similar uptake mechanism to fluvastatin, being partly simple diffusion and specific active transport, whereas pravastatin K_m 32.3 μM shows only a specific OAT Na^+ independent mechanism with little passive diffusion in hepatocytes. Hepatocellular uptake of fluvastatin was inhibited by pravastatin, indicating a common transport system. In human aortic endothelial cells uptake of fluvastatin was by simple non-specific diffusion, via two binding sites with dissociation constants of 13.7 and 165 μM (calculated from the difference between uptake at 37° and 4° although it is possible that the 4° data may be partially due to reduced membrane fluidity). The drugs uptake into the cells was shown to be predominantly the unchanged drugs. [25] The *estimated* binding free energy ΔG_{bind} for fluvastatin-OATP1B1 is -6.3 kcal/mol, and the value for the non-specific facilitated diffusion, presumably via a passive carrier protein, is about -6.9 kcal/mol at 37°C. Table 2 shows that the desolvation energy for fluvastatin anion, acid and lactone is 76.4, 25.6 and 14.2 kcal/mol respectively. The lipophilicities are -32.5, -17.9, -16.4, and the dipole moments are 36.6, 9.3 and 1.5 respectively. The molecular volumes are fairly similar. These data indicate that active transport by OATP1B1 or by a passive carrier protein are more likely to involve the neutral acid or more likely the neutral lactone forms.

Basolateral uptake of rosuvastatin in Caco-2 cells has a saturable and non-saturable component with an apparent K_m 4.2 μM , a saturable maximum flux J_{max} 6.1 $\text{pmol}\cdot\text{min}^{-1}\cdot\text{mgprotein}^{-1}$, and a facilitated diffusion coefficient 1.0 $\mu\text{l}\cdot\text{min}^{-1}\cdot\text{mgprotein}^{-1}$. [22]

The uptake of rosuvastatin by OATP1B1 in hepatocytes is strongly inhibited by gemfibrozil IC_{50} 4.0 μM and cyclosporin 2.2 μM . In healthy people, this led to an average exposure increase to rosuvastatin of 1.9 fold for gemfibrozil, and a very large 7.1 fold total higher exposure for cyclosporin. Other statins show large AUC fold increases when co-administered with gemfibrozil or cyclosporin [6,26,27] It is unclear which species of statin were transported in the inhibition studies. There is now a 2012 FDA and European Medicines Agency requirement to consider if an investigational drug is a substrate of OATP1B1 or OATP1B3 during clinical trials, particularly if hepatic elimination is > 25%. The current medication guide for Crestor, rosuvastatin, warns that co-administration medications such as cyclosporin which are inhibitors of OATP1B1 transporters, may lead to increased plasma levels of rosuvastatin and increased risk of myopathy.

2.4 CYP metabolism:

As shown in Table 1, all statins undergo CYP3A4 metabolism with the exception of pravastatin, pitastatin and rosuvastatin, and fluvastatin undergoes CYP2C9 metabolism. Due to rapid metabolism in the gut and liver, the system bioavailability is generally low, except for cerivastatin and pitastatin.

The statin acids and particularly the lactones are considered to have high first pass metabolism, but the situation is complicated in blood plasma by the high binding to human serum albumin (HSA) for all statins, except pravastatin. The bioavailability of the statins (see Table 1) is complicated by the pH dependent equilibrium between acid and lactone forms, and the HSA binding. It is safe to conclude that it is unclear what are the active species for each statin, particularly when categorized into active species in blood serum, the species being transported across the cell membrane, both uptake and efflux, and the species being metabolized by CYP enzymes intracellularly, and the relationship between first pass metabolism and bioavailability. Where a DDI can occur, or where pre-existing medical conditions exist, eg diabetes, kidney transplants etc, the situation becomes even more complex. The more bioavailable the statin form, the more the higher the systemic concentration, and the more likely a DDI can occur.

The metabolic clearances of the acid and lactone forms of atorvastatin, simvastatin, cerivastatin, fluvastatin, rosuvastatin and pitastatin from human hepatic microsomes at 2.5 μM are 26, 28, 21, 33, 1, 3 and 1892, 1959, 622, 226, 71, 5 ($\mu\text{L}/\text{min}/\text{mg}$ protein) respectively. [17,18] The CL_l lactone/acid clearance ratios are 73, 70, 30, 7, 71, 2 respectively. Metabolic clearance is predominantly driven by cellular transport and CYP oxidation, and the acid and lactone metabolism was dependant on CYP3A4 with the exceptions of CYP2C9 being the dominant enzyme for fluvastatin and pitastatin, and CYP2C8 and CYP3A4 being of equal importance for cerivastatin, and rosuvastatin acid being mostly excreted unchanged. *The CYP3A4-mediated metabolism of the lactone forms is a common metabolic pathway for statins.* The metabolic oxidation by the CYP enzymes leads to metabolites which are effluxed out of the cell and excreted. The situation is further complicated by the observation that CYP metabolites of lactones can hydrolyse to acids. Some drugs are known to competitively bind to these CYP enzymes when present with statins, which have a narrow therapeutic index, leading to reduced oxidative metabolism of the statins, and increasing statins concentrations with concomitant side effects.

The low CL_l lactone/acid clearance ratio for pitastatin, 2, is related to the fact that it is only slightly metabolized by the CYP2C9 enzyme, hence its high bioavailability of 60%. Pitavastatin is minimally metabolized and most of the bioavailable fraction of an oral dose is excreted unchanged in the bile and is reabsorbed by the small intestine ready for enterohepatic recirculation. Its metabolite is the lactone. Neither pitavastatin nor its lactone form, have inhibitory effects on CYP enzymes, and CYP3A4 inhibitors have no effect on pitavastatin concentrations. Moreover, P-glycoprotein-mediated transport does not play a major role and pitavastatin does not inhibit P-glycoprotein activity. Pitavastatin is transported into the liver by several hepatic transporters but OATP1B1 inhibitors have relatively little effect on plasma concentrations compared with other statins. Pitavastatin has minimal drug-food and drug-drug interactions making it a treatment option in the large group of dyslipidaemic people who require multidrug therapy. Pitastatin has significant LDL-C-lowering efficacy at low doses compared to other statins. [28,29]

Uptake (solute carriers, SLC families) and efflux (ATP binding cassette, ABC families) transporters are major determinants of statin therapeutics, and along with the efficacy of

metabolic disposal by CYP enzymes, govern the therapeutic index of these drugs. [5, 30,31] As previously discussed in section 2.4 and 2.6 the cellular transport of the statins is a competitive process between passive facilitated diffusion and active transport. However a critical factor which pre-determines the uptake of statins from the blood is the known reversible binding interaction of statins with blood proteins, particularly human serum albumin (HSA). HSA is the principal carrier of unesterified free fatty acids in serum and its major functions include maintaining osmotic pressure, transporting endogenous and exogenous ligands. At physiological pH levels, HSA is negatively charged. It is known that all the approved statins in clinical use are highly bound to HSA (> 90%) with the exception of pravastatin which is ca. 50% bound (Table 1). Since all statins, except lovastatin and simvastatin, which can be given in the lactone form (but then bio-transformed to the acid forms), are administered in the acid form (rosuvastatin, atorvastatin, fluvastatin, simvastatin can also be given in the anionic salt forms) which transform to the acid form at physiological pH), it can be assumed that the acid forms are the species reversibly binding to HSA. As it is known that statin acids can be in equilibrium with the lactone form in blood serum, it is likely that either neutral species could be the transported species that crosses the cell membrane by passive or active transport. [9,32-36]

In a study of the acid and lactone forms of atorvastatin, lovastatin and simvastatin (and pravastatin in acid form), it was found that the **IC₅₀ values** (transfected canine kidney cells) for the **uptake transporter OATP1B1 were 3-7 times lower for the acid forms than the lactone forms**, but for the **efflux transporters MDR1, MRP2, Mrp2, the IC₅₀ values for the lactone forms were up to 10 times lower**. [34] Bidirectional transport studies of atorvastatin, fluvastatin, rosuvastatin, lovastatin and simvastatin in Caco-2 cell monolayers showed that atorvastatin, fluvastatin and rosuvastatin acids had much higher basolateral to apical than apical to basolateral rates. However the lovastatin and simvastatin lactones showed little efflux, and a substantial amount of the lactone was converted into the acid form, but there was little efflux of the newly created acid form. [37] Oral administration of atorvastatin, lovastatin and simvastatin in acid form in humans has been shown to produce both acid and lactone forms in plasma, which is opposite from that shown in mice after subcutaneous injection.[35]

In a study of the CYP metabolism and DDI interactions of the acid and lactone forms of atorvastatin, it was found that the lactone pathway is the major determinant of metabolic clearance and DDI. Metabolism by CYP3A4/5 of both statins resulted in para- and ortho-hydroxy metabolites, and the lactone had a higher affinity to CYP3A4 than the acid form, with the K_m values for para-hydroxyatorvastatin 1.4 and 25.6, and for ortho-hydroxyatorvastatin 3.9 and 29.7 μM respectively. It is noted that the binding affinity of the lactone is more than 5 times those for lovastatin and simvastatin. CYP dependent metabolism of the lactone was 83 fold higher than for the acid (para-hydroxylation), and was 20 fold higher for ortho-hydroxylation. Atorvastatin lactone was a potent inhibitor of the metabolism of the acid by human liver microsomes, K_i 0.9 μM , but the atorvastatin acid was a poor inhibitor of the metabolism of the lactone, K_i 90 μM . The experimental binding energies for the lactone and acid in CYP3A4 were 1.3 and 1.8 kcal/mol for ortho- and para-hydroxylation. [38] It has been shown that DDI between lovastatin and simvastatin (administered as the lactone) and itraconazole led to a > 20 fold and 18.8 fold increase in the area under the curve AUC values, whereas the same DDI with atorvastatin led to a 3.2 fold increase in AUC. [13] This observation is consistent with the higher binding affinity of atorvastatin compared to those for lovastatin and simvastatin

High plasma levels of the lactone forms have been observed in patients with statin induced myopathy. In primary human skeletal muscle cells, atorvastatin lactone showed a 14-fold, fluvastatin lactone a 26-fold, pravastatin lactone a 23-fold, and simvastatin lactone a 37-fold higher potency to induce myotoxicity compared to their corresponding acid forms. [32]

The OATP1B1 hepatic transporter inhibitor, rifampin, affects the kinetics of atorvastatin and its metabolites in humans. An intravenous dose of rifampin significantly increased the plasma concentration of atorvastatin acid by 6.8 fold and that of the metabolites 2-hydroxy-atorvastatin acid and 4-hydroxy-atorvastatin acid by 6.8 and 3.9 fold, respectively. The levels of the lactone forms of atorvastatin, 2-hydroxy-atorvastatin and the metabolite 4-hydroxy-atorvastatin, were also significantly increased, but to a lower extent. The data confirm that OATP1B transporters represent the major hepatic uptake systems for atorvastatin and its active metabolites. [39]

Gemfibrozil can modulate the pharmacokinetics of statins more via inhibition of statin hydroxyl acid glucuronidation than via inhibition of CYP3A4-mediated oxidation. Gemfibrozil is not a strong inhibitor of CYP3A4. Glucuronidation is a common, metabolic pathway for the conversion of active open acid forms of several statins (including atorvastatin and rosuvastatin) to their lactone form. The lactone form, in turn, plays a critical role in the subsequent statin metabolism catalyzed by CYP3A4. Consistent with the severe interactions reported with gemfibrozil, cerivastatin was shown to be more susceptible than simvastatin and atorvastatin to metabolic glucuronidation with gemfibrozil. [40]

Gemfibrozil co-administration with statins results in AUC increases for simvastatin, lovastatin, pravastatin (all in the 2-3 fold range), cerivastatin 5.6 fold, and atorvastatin about 1.3 fold. The glucuronidation of atorvastatin yields an acyl glucuronide which undergoes pH dependent lactonization to atorvastatin lactone, K_m 12 μ M, K_i 75 μ M. Atorvastatin lactone gives an ether glucuronide, K_m 2.6 μ M. Gemfibrozil, fenofibrate, and fenofibric acid inhibited atorvastatin lactonization with IC50 values of 346, 320, and 291 μ M respectively. Based on unbound fibrate concentrations at the inlet to the liver, these data predict a small increase in atorvastatin AUC (1.2 fold) after gemfibrozil coadministration and no interaction with fenofibrate. [41]

2.5 Effect of statins on patients with pre-existing medical conditions:

In a study of the effect of atorvastatin acid and lactone in patients with (and without) diabetes mellitus, the effect of diabetes on the biotransformation of atorvastatin acid, both in vivo in nondiabetic and diabetic renal transplant recipients, and in vitro in human liver samples from nondiabetic and diabetic donors was examined. In diabetic patients, the plasma concentration of atorvastatin lactone was significantly higher than that of atorvastatin acid throughout the 24-hour sampling period. Diabetic patients have 3.6 times lower apparent total clearance than nondiabetic patients. The concentration of atorvastatin acid remaining in the microsomal incubation was not significantly different between nondiabetic and diabetic liver samples, whereas the concentration of atorvastatin lactone was significantly higher in the samples from diabetic donors. CYP3A4 was responsible for the biotransformation of atorvastatin lactone. [42]

In a similar study of the effects of atorvastatin acid and lactone in patients with the kidney transplants demonstrated a significant reduction of clearance of atorvastatin lactone compared

to the acid form, and thus they may be at higher risk of developing myotoxicity, since atorvastatin lactone is known to increase myotoxicity. [43]

2.6 Clearance and elimination of statins:

The metabolic clearance rate is the volume of biological fluid completely cleared of drug metabolites as measured in unit time. Elimination occurs as a result of metabolic processes in the kidney, liver, saliva, sweat, intestine, heart, brain, or other sites. Because statins are highly bound to blood serum proteins, clearance is the elimination of (free statins + plasma protein bound statins). The clearance rate for protein bound statins is lowered by protein binding, which is >90% for all statins except pravastatin at ca. 50% (Table 1). Clearance in the kidney is mainly by filtration, whereas in other sites, clearance is by membrane transport proteins. Clearance in the liver is mainly by filtration through the sinusoidal system.

It has been shown that the rate determining process in the hepatic elimination of statins (pravastatin, pitastatin, atorvastatin and fluvastatin) in rats and humans is the uptake process. This finding using cryopreserved human hepatocytes and a rat scaling in vitro factor (based on the finding that scaling factors for P450-mediated metabolism are preserved in humans and rats). The in vivo uptake clearances were pravastatin 1.44, pitavastatin 30.6, atorvastatin 12.7, and fluvastatin 62.9 (ml/min/g liver), compared to clinically determined values for intrinsic clearance of 0.8-1.2, 14-35, 11-19, and 123-185 respectively. The OATP transporters were the saturable uptake agents.[44] These results are similar to those observed in Corning Transportocells OATP1B1 where a linear relationship between the intrinsic uptake clearance for the statins and clinically observed hepatic uptake clearances was observed. [22]

Table 3 shows the metabolic clearance for the acid and lactone statins forms from human hepatic microsomes. The very high lactone:acid clearance ratio for all statins (except pitastatin at 2) reinforces the various studies described in section 2.4 and 2.6.

2.7 Cytotoxicity of statins:

The cytotoxicity of statins ranges from their use as anti-cancer agents [45,46] to extreme side effects, particularly cardiomyopathy. The withdrawal of cerivastatin from the market is a well known example. Since statins inhibit cholesterol synthesis mainly in the liver, it is not surprising that elevations in the liver enzymes ALT and AST can occur. The principal safety issue with statins is the potential to cause acute liver failure. Four types of hepatic syndromes need to be considered: acute liver failure, hepatitis, cholestasis and transaminitis.

The cardiac effects of statins are subject to controversy, and the mechanism of their uptake into the human heart is unknown. The expression of OATP2B1 was analyzed in 46 human atrial and 15 ventricular samples, including samples from hearts with dilated cardiomyopathy and hearts with ischemic cardiomyopathy. OATP2B1 is a high-affinity uptake transporter for atorvastatin (K_m 0.2 μ M) and is expressed in the vascular endothelium of the human heart, suggesting its involvement in cardiac uptake of atorvastatin. Simvastatin (lactone) was not transported by OATP2B1. Patients who had taken atorvastatin exhibit decreased OATP2B1 messenger ribonucleic acid expression compared with patients with no statin treatment. [47]

In primary human skeletal muscle cells, atorvastatin lactone showed a 14-fold, fluvastatin lactone a 26-fold, pravastatin lactone a 23-fold, and simvastatin lactone a 37-fold higher

potency to induce myotoxicity compared to their corresponding acid forms.[32] Statin lactones have been found to be more potent inducers of myopathy than the acid forms by a factor of about three times, a result attributed to strong inhibition (up to 84%) of mitochondrial complex III (CIII) activity. The Qo binding site of CIII was identified as the off-target of the statin lactones. [48]

In a study of the relationship between pravastatin- and rosuvastatin-induced cytotoxicity and medium pH using human prototypic embryonal rhabdomyosarcoma cell line (RD) and rat myoblast cell line (L6) as a model of in vitro skeletal muscle, it was found that rosuvastatin cytotoxicity, reduction of cell viability, morphological changes and caspase activation at acidic pH (pH 6.8) were significantly greater than those at neutral pH (pH 7.4). Rosuvastatin accumulation at acidic pH was greater than that at pH 7.4. Medium pH had no effect on pravastatin accumulation or cytotoxicity. Rosuvastatin cytotoxicity at acidic pH is associated with increasing intracellular accumulation of rosuvastatin. [49]

A study of simvastatin, lovastatin and pravastatin induced cytotoxicity on B16.F10 murine melanoma cells in vitro has shown that only simvastatin and lovastatin exhibited cytotoxicity. The cytotoxic actions were mainly based on the suppressive actions on hypoxia-inducible factor 1 α expression and nonenzymatic antioxidant levels, as well as because of the inhibition of superoxide dismutase activity in B16.F10 melanoma cells. [50]

3. Results

3.1 Energetics of cellular membrane transport processes:

To gain an understanding of the differences between the passive diffusion and active transport mechanisms, an understanding of the relative energies involved is required. Passive diffusion, including facilitated diffusion (which uses a trans-membrane spanning protein) does not require any external energy source to drive the transport, whereas active transport which uses a transporter to move a substrate against the electrochemical gradient requires an energy source, usually ATP. The free energy of ATP hydrolysis ΔG_{ATP} is about 14.3 kcal/mol under cellular conditions. Transport systems can use the energy contained in trans-membrane gradients to add to the energy from ATP or GTP cycles. The total electrochemical potential gradient averages about -170 mV for normal cell membranes. [51] The cell membrane potential is about -40 to -80 mV (about 1-2 kcal/mol). The transport of drugs by active transporters requires considerable energy. For example, the energy expended by cells to maintain the concentration gradients of Na⁺, K⁺, H⁺, and Ca²⁺ across the plasma and intracellular membranes in nerve and kidney cells, requires about 25 percent of the ATP produced by the cell for ion transport; and in human erythrocytes, up to 50 percent of the available ATP is used for this purpose. [52] Cations are known to generally more easily be transported across the cell membrane than anions. Little is known about the free energy required to transport large neutral and charged molecules (drugs, proteins etc) across cell membranes. The transport of small bare neutral and charged molecular species across the membrane electrical gradient can in principle be calculated using the Nernst equations, but other factors such as desolvation, binding energy between the substrate and transporter, and conformational changes required for larger molecules and/or the protein transporters during binding can have very large energy penalties, which would require many ATP or GTP energy releases to drive active transporters. [20,52,53] The experimental binding energies between substrates and transporters are roughly in the region of -8 to -20 kcal/mol (for instance the binding energy of the aspartate transporter Glt_{Ph} (Pyrococcus horikoshii), is -12 kcal/mol

[54], while the activation energy for the temperature dependent transport reaction of GlpT-G3P phosphate in E.coli is 8.4 kcal/mol.[55] Typical antibody-antigen dissociation constants are characteristically near 10^{-8} M (free energy of binding is -11 kcal/mol), ATP binds to myosin with a dissociation constant of 10^{-13} M (free energy of binding is -17.9 kcal/mol) and biotin binds (effectively irreversibly) to avidin, a protein found in egg white, with a dissociation constant of 10^{-15} M (free energy of binding is -20.6 kcal/mol), which is the strongest known (non-covalent) small molecule-protein binding energy. [56,57] The binding energies of statins with HMG-CoA reductase are in the region of -9 to -12.5 kcal/mol.[58] The loss of translational and rotation entropy of the small molecule ligands on binding can contribute a penalty up to about 12 kcal/mol towards the total binding energy. The bound conformation of the ligand is usually higher than the free ligand, by about an average of 4-5 kcal/mol, with 10% of ligands having a higher energy of up to 9 kcal/mol. [59]

To get an understanding of the magnitude of free energy changes involved in passive and active transport, an examination of the known uptake mechanism of D- and L-glucose by human red blood cells is instructive. The facilitated diffusion of D- and L-glucose by the Glut1 transporter is highly stereospecific with the D-isomer having a K_m of 1.5 mM, compared to the L-isomer value of >3000 mM. This corresponds to a binding free energy difference of greater than -4.7 kcal/mol. There is a single trans-membrane spanning protein with a single site, accessible from inside or outside of the membrane, and the glucose causes a conformational change of the protein transporter. [60] If the concentration of D-glucose inside the cell was say 0.05 mM, and the extracellular concentration was say 5.0 mM, then at 310K the free energy ($\Delta G = RT \ln[\text{intra-cellular}]/[\text{extra-cellular concentration}]$) of *non-saturable facilitated transport* would be about -2.9 kcal/mol. However if *active saturable transport* was required to say translocate D-glucose against the concentration where the extracellular concentration was say 0.005 mM and the intracellular concentration was say 5.0 mM, then the ΔG required is about +4.3 kcal/mol. It is known that the active transport of glucose requires two Na^+ to facilitate active transport: if the extracellular and intracellular concentrations of Na^+ were say 140 and 10 mM (typical values for mammalian cells), then the ΔG available is 2×-1.6 kcal/mol, roughly -3.2 kcal/mol. The electrical gradient across the membrane can be calculated from the Nernst equation ($\Delta G = z.F.V_m$ where z is the ionic charge, F is the energy released as one mole of charge moves down a voltage gradient, and V_m is cellular membrane potential, about 70mV for mammalian cells. For two Na^+ this translates to roughly -3.2 kcal/mol, so the total *electrochemical gradient* to move glucose against a steep concentration gradient (ie from 0.005 outside to 5.0 mM inside) is about -3.2 plus -3.2 kcal/mol which is sufficient to overcome the +4.3 kcal/mol required. The Na^+ gradient across the plasma membrane is created by the active transport of Na^+ out of the cell by the Na^+/K^+ ATPase pump.

So from Table 2, it can be seen that desolvation energies of substrates prior to binding with active transporters, and the biophysical properties of the ligand species being transported, particularly the lipophilicity, dipole moment, and molecular size, can be comparable in magnitude to cellular transport energies and transporter binding energies, without even considering transporter protein conformational energy changes. The desolvation data for the anions, acids or lactones has strong implications for what species are actually being transported, since the penalty for the anionic species are very high compared to the neutral species. For example, in Table 2 it can be seen that the desolvation energies for the anionic statin species range from 74-104 kcal/mol, while the acid species range from 25-38 kcal/mol, while the lactones range from 14-32 kcal/mol. The difference between the neutral species and the anionic species is very large, considering the range of energies involved in transport

processes discussed in the above two paragraphs. While it is unknown what degree of desolvation is required for active and passive transcellular processes, the desolvation energy gap is simply too large for charged species. In addition, substantially desolvated charged species have a strong electrostatic effect, which can be repelled by hydrophobic protein surfaces, but possibly attracted to protein sites of the opposite charge. The dipole moment is a measure of electrostatic polarity, Since these neutral and charged species are in equilibrium in blood plasma, *it is likely that the neutral species, most probably the lactones, are the actual species being preferentially translocated by the OATP transporters*, rather than the anionic species.

3.2 Redox metabolism by CYP enzymes

The metabolic transformation of drugs, including the statins, in the mitochondria can be found experimentally *in vitro*. The oxidation-reduction mechanism is well known, involving electron transfer from a heme centred moiety within the CYP enzyme to the substrate. A useful theoretical means of predicting this redox mechanism is the reduction potential of the substrate. The electron affinity (EA) is a measure of the acceptance of an electron by a molecule, and so the EA is a measure to the ease of reduction (acceptance of an electron) to form metabolites such as hydroxylated species etc. [61] Table 2 shows the calculated EA for the various statin anion, acid and lactone species in water. It can be seen that there are some very significant differences for the same statin when in the anion, acid, and lactone forms. The use of EA as an indicator of ease of reduction is only applicable to a series of substrates which have a strong chemical structural similarity, and strong linear relationships have been observed between reduction potentials and substrate reactivity properties. These relationships appear to be present in the three statin forms in Table 2, with the one exception of rosuvastatin lactone where the EA value is abnormally higher than the other statin lactones, and appears to be related to the lower charges on the atoms of the (unique to the statins) methanesulphonate group. This value was omitted from development of the statistical models described below.

The electrochemical redox properties of the statins have been determined in a number of studies. Generally, with the exception of pravastatin, which can show reversible redox behaviour, the statins undergo irreversible reduction in acid and alkaline buffers, and often show linear relationships between peak reduction potentials and pH. Irreversible redox behaviour is a result of the formation of reduced species, and for simvastatin and lovastatin lactones, probably being the formation of the acid species at acid pH, and for other statins, tested as the anionic salts, probably hydroxylation. The acid form of the anionic salt would predominate at acid pH levels. The statin lactones are known to be H^+ catalytically hydrolysed at acid pH in blood serum to the acid form, but the equilibrium can be pushed to the lactone side at pH 6. [8] However the acid:lactone equilibrium may be different in the absence of serum proteins, as in the electrochemical studies, where it was observed that the peak reduction potential of simvastatin was independent of pH above pH 5 (up to pH 11), [62] which probably suggests that it is the acid form of simvastatin that predominates above pH 5 to 7, and the anionic form at $> pH 7$. The following peak reduction potentials (relative to Ag/AgCl reference electrode) at $\sim pH 5$ were simvastatin lactone 1.12V [62] lovastatin lactone 1.49V pH 6 [63,64] fluvastatin 0.64V [64] rosuvastatin 1.18V [65], pravastatin 1.32V [63] atorvastatin 0.92V [66] pitastatin 1.21V pH 6. [67] The absolute reduction potential were calculated according to the method of Topol 2001 to compare with the experimental electrochemical reduction potentials and the EA values calculated in water. It was found that the trends were similar in all cases, but exceptions were outliers from the general trend,

notably rosuvastatin lactone which has an anomalously high EA. The electrochemical values for fluvastatin and pravastatin acid seemed much lower than the other experimental values, which reflect the chemical equilibria at ~pH 5.

The biophysical data of a number of drugs that cause DDI with statins are listed in Table 2. It can be seen that the neutral species of these drugs have similar properties as the neutral acid or lactone forms of the statins, so easily fit into the therapeutic window for these drugs. It is suggested that such a criteria is a useful screening tool for possible DDI with statins. The very high desolvation energies for the charged species would effectively preclude these forms of the drugs from being active in transport processes, and hence any interaction with CYP enzymes. Comparison of the fibrates, gemfibrozil, fenofibrate, and fenofibric acid shows that they have similar properties with the exception of their EA values, 0.6, 2.6, 2.4 eV respectively, which indicates that the species would have similar cell transport properties, but the neutral form of gemfibrozil would passively and actively transport much faster. The higher EA for fenofibrate and fenofibric acid means that these drugs would be reduced faster than gemfibrozil by glucuronidase or CYP enzymes, and thus less likely to inhibit the enzymes. This data is consistent with the observed clinical effects where gemfibrozil can cause serious DDI with statins, but fenofibrate does not. [40,41] Gemfibrozil and its glucuronide inhibit CYP2C8 and OATP1B1. [4-6] Gemfibrozil has also been shown to inhibit the OATP1B1 uptake of rosuvastatin. [26]

Statin DDI involving warfarin, niacin anions, verpamil or mifefradil are more likely to occur with the neutral species rather than their co-equilibrated ionic species based on the very large desolvation penalties required for the ionic species to interact with transporters or CYP enzymes. Cyclosporin and digoxin are unusual in that these neutral drugs have very large desolvation values, which may indicate that their main effect is to inhibit statin transport. Digoxin is a substrate of OATP1B3 or possibly another transporter [68,69] and cyclosporin is reported to passively diffuse (presumably facilitated diffusion in view of the large molecular size of cyclosporin) into rat hepatocytes and has a membrane fluidising effect. [70]

It is noted that coenzyme Q10, ubiquinone, fits into this therapeutic window, and its high EA would suggest that it would be effective in mitigating any redox processes such as reactive oxygen species (ROS) that could be involved in cytotoxic oxidative stress side reactions. CoQ10 plays an important role in mitochondrial energy production by participating in the electron transport chain, preventing oxidative stress, and regenerating active antioxidant vitamins C and E in mitochondria. Statins are known to decrease the cellular levels of glutathione, and increase the levels of oxidised glutathione, an indicator of oxidative stress. [71] This observation gives some basis to the moderate use of coenzyme Q10 supplements as being useful in offsetting statin side effects such as myalgia. [2] Statin treatment reduces circulating levels of CoQ10, largely because CoQ10 is carried in LDL particles, but its effect on intramuscular levels of CoQ10 remains ambiguous. [72]

Endothelial dysfunction and the imbalance between nitric oxide (NO) and reactive oxygen species production in the vascular endothelium are important early steps in atherogenesis, a major socioeconomic health problem. Statins can modify endothelial function and affect atherogenesis by regulating the redox state in the vascular endothelium. [73] EA data may be easily accessible indicators of redox properties.

The pleiotropic actions of hydroxymethylglutaryl CoA reductase inhibitors (statins) include anti-inflammatory and antioxidant actions. Statins promote potent systemic antioxidant

effects through suppression of distinct oxidation pathways. The major pathways inhibited include formation of myeloperoxidase-derived and nitric oxide– derived oxidants, species implicated in atherogenesis. [74] The EA of the statins could be a useful biophysical indicator of the anti-inflammatory and antioxidant of statins, as they are a direct measure of the redox capacity of the statins.

3.3 Active and passive transport models for statins:

A passive diffusion permeation model for atorvastatin, fluvastatin, cerivastatin, pravastatin, pitastatin, lovastatin, rosuvastatin statins based on passive permeation diffusion data for the Corning Transportocells control cells [22] has been developed. This model is based on the similar model developed for the drug permeability of the blood brain barrier, and incorporates four independent variables, the free energy of water desolvation of the drug, drug lipophilicity based on the free energy of solvation in n-octane, the dipole moment in water, and molecular volume in water. [20] The best fit of the passive diffusion rate was for the lactone form, where equation 1 shows a positive correlation with the desolvation free energy in water, dipole moment and molecular volume, and a negative correlation with the lipophilicity. The magnitude of the effects (the coefficients of the independent variables) are highly significant for all variables other than the molecular volume where the standard error is larger than the coefficient. Equations 1-3 can better represented as depending only on desolvation, lipophilicity and dipole, as the molecular volume term is not significantly different from zero. The small coefficients for the molecular volume variable are about the same or smaller than their standard errors, indicating a zero dependency. The standard error of the estimate (the dependent variable, the diffusion rate) of 4.2 compares with the range of rates from 0.17 to 88.4 $\mu\text{L}/\text{mg}/\text{min}$ (rosuvastatin 0.17, pitavastatin 20.7, pravastatin 0.61, simvastatin acid 13.8, fluvastatin 31.4, atorvastatin 6.26, cerivastatin 88.4, lovastatin acid 50). The corresponding equations for the acid and anionic statins are shown in equations 2 and 3 which have lesser statistical precision than the equations for the lactones. Based purely on the degree of statistical fit, this data implies that the lactone form is the likely form that permeates the cell membrane by passive diffusion. Analysis of the trend of the coefficients does show the relative magnitudes of the independent variables, which can shed mechanistic light on the passive diffusion process.

Comparison of the *trend* of the coefficients for equations 1-3 show that:

- (a) for the statin lactones, it is the dipole moment (the electrostatic interaction between the statin and the protein transport channel that is involved in passive facilitated diffusion, or for the less likely simple diffusion, the interaction between the statin and the cell membrane) that mainly determines the passive diffusion rate
- (b) for the statin acids, it is the lipophilicity (the hydrophobic interaction) and to a lesser extent, the dipole that determines the passive diffusion rate
- (c) for the anionic statins, it is the desolvation energy and the dipole moment that determine the passive diffusion rate. This finding is consistent with the likelihood that the anionic form is unlikely to permeate the cell membrane based on the magnitude of the desolvation energies required for this form (see discussion in section 3.1). The larger contribution from the electrostatic or dipole interaction is consistent with the greater negative charge on the anions, compared with the lactones or acids.
- (d) the zero dependence of equations 1, 2, 3 on molecular size of the statins indicates that the mechanism of statins passively diffusing through the cell membrane is via a *facilitated diffusion mechanism* where the statins are passively transported down a trans-membrane protein channel where the size of the channel can accommodate the molecular volumes of

all statins which vary from ca. 280 to 440 cm³/mol. If the passive diffusion process was a purely simple diffusion through the cell membrane, then a correlation would be found with molecular size (as well as desolvation, lipophilicity and dipole moment) as is found with smaller drugs. [20]

Equation 1a which shows the passive diffusion rates for statin lactones in rat hepatocytes can be compared directly with eq 1 showing the in vitro passive diffusion rates for the same lactones. It can be seen that the relative magnitude and signs of the coefficients are similar (though the in vitro eq 1 is ca 10 times more sensitive than the in vivo eq 1a), with the exception of the dipole moment which is relatively much larger in the rat hepatocyte than the in vitro case.

An analysis of the published K_m rat data from the same experimental study is available for rosuvastatin 7.5, pitastatin 6.3, pravastatin 30.0, fluvastatin 37.6, atorvastatin 4.0 and cerivastatin 7.0 μM . [75] This data has been analysed in equations 4 and 5. Equation 4 (lactone form) is more statistically robust than equation 5 (acid anion form) which might suggest that it is the lactone form that is preferentially taken up by the active OATP transporter, and it is the lipophilicity and dipole moment that governs the thermodynamic binding affinity between the statin lactone and the OATP transporter. This observation is consistent with an essentially electrostatic interaction plus a hydrophobic interaction between the polar and non-polar portions of the statin and the transporter protein respectively. The K_m correlation with the lipophilicity and dipole moment is consistent with a substrate-protein equilibrium where the substantially desolvated statin substrate lies within, and interacts with the protein environment, and outside the bulk blood plasma environment. If equation 4 and 5 are valid then a substantial desolvation must occur before the statin can enter the protein environment and bind to it, and that the hydrophobic interaction is counterbalanced by the electrostatic interaction in the total binding interaction.

Comparison of the trend of the coefficients for equations 4 and 5 show:

- (a) desolvation required for active transport of statins by the rat OATP is larger for the anions than the lactone species, but the relative contributions by the other three variables remains fairly similar
- (b) for the active transport of the lactones, the dipole or electrostatic interaction and to a lesser extent the lipophilicity or hydrophobic effect determines the binding interaction between the statin lactones and the OATP transporter
- (c) for the active transport of the acid anions, the dipole or electrostatic interaction and the lipophilicity or hydrophobic effect determine the binding interaction, with a smaller contribution from the desolvation energy.

However these *indicative* equations (which are not strongly robust in view of the limited number of experimental data points) can only be supportive evidence, but data from other literature studies (as discussed in sections 2.1, 2.4, 2.6) does support the hypothesis that the lactone form is the likely form that is responsible for the majority of the passive diffusion and active transport by OATP transporters. It is possible that both neutral acid and lactone forms are competitively transported across the cell membrane. In view of the molecular volume or size of these statins, it is likely that the transport mechanism is a facilitated passive diffusion process. From equation 4, it can be estimated that the average polar (electrostatic) interaction between the statins and the OATP transporter protein is *ca. 2 times as strong* as the hydrophobic binding interaction.

For the statin lactones: Corning Transporto cells, [Li 2014] Equation 1

$$\text{Passive diffusion rate} = -2.96\Delta G_{\text{desolvation}} + 7.51\Delta G_{\text{lipophilicity}} + 2.26 \text{ Dipole Moment} + 1.11 \text{ Molecular Volume} - 143.53$$

Where $R^2 = 0.997$, $SEE = 4.17$, $SE(\Delta G_{\text{desolvation}}) = 0.30$, $SE(\Delta G_{\text{lipophilicity}}) = 1.03$, $SE(\text{Dipole Moment}) = 0.68$, $SE(\text{Molecular Volume}) = 0.12$, $F=80.91$, $\text{Significance}=0.082$, $no=6$

For the statin lactones: rat hepatocytes, [Shitara 2013] Equation 1a

$$\text{Passive diffusion rate} = -0.38 \Delta G_{\text{desolvation}} + 1.23 \Delta G_{\text{lipophilicity}} + 2.90 \text{ Dipole Moment} + 0.15 \text{ Molecular Volume} - 25.92$$

Where $R^2 = 0.961$, $SEE = 4.97$, $SE(\Delta G_{\text{desolvation}}) = 0.16$, $SE(\Delta G_{\text{lipophilicity}}) = 1.23$, $SE(\text{Dipole Moment}) = 0.81$, $SE(\text{Molecular Volume}) = 0.14$, $F=6.141$, $\text{Significance}=0.290$, $no=6$

Eq 1 (in vitro) and eq 1a (in vivo) are similar, indicative of similar (probably facilitated) diffusion processes.

For the statin acids: Corning Transporto cells, [Li 2014] Equation 2

$$\text{Passive diffusion rate} = -1.05 \Delta G_{\text{desolvation}} + 10.47 \Delta G_{\text{lipophilicity}} + 3.54 \text{ Dipole Moment} + 0.33 \text{ Molecular Volume} + 98.87$$

Where $R^2 = 0.881$, $SEE = 20.06$, $SE(\Delta G_{\text{desolvation}}) = 2.69$, $SE(\Delta G_{\text{lipophilicity}}) = 3.73$, $SE(\text{Dipole Moment}) = 4.83$, $SE(\text{Molecular Volume}) = 0.16$, $F=3.21$, $\text{Significance}=0.18$, $n=8$

For the statin anions: Corning Transporto cells, [Li 2014] excluding cerivastatin outlier equation 3

$$\text{Passive diffusion rate} = 0.66 \Delta G_{\text{desolvation}} + 0.29 \Delta G_{\text{lipophilicity}} - 0.95 \text{ Dipole Moment} - 0.28 \text{ Molecular Volume} + 97.45$$

Where $R^2 = 0.919$, $SEE = 8.95$, $SE(\Delta G_{\text{desolvation}}) = 0.54$, $SE(\Delta G_{\text{lipophilicity}}) = 0.32$, $SE(\text{Dipole Moment}) = 1.37$, $SE(\text{Molecular Volume}) = 0.22$, $F=5.69$, $\text{Significance}=0.155$, $no=7$

For the active transport of statin lactones by OATP transporter, rat hepatocytes, [Shitara 2013]: Equation 4

$$K_m = -0.33 \Delta G_{\text{desolvation}} + 2.26 \Delta G_{\text{lipophilicity}} - 3.77 \text{ Dipole Moment} - 0.03 \text{ Molecular Volume} + 96.3$$

Where $R^2 = 0.972$, $SEE = 5.40$, $SE(\Delta G_{\text{desolvation}}) = 0.39$, $SE(\Delta G_{\text{lipophilicity}}) = 1.34$, $SE(\text{Dipole Moment}) = 1.88$, $SE(\text{Molecular Volume}) = 0.15$, $F=8.76$, $\text{Significance}=0.24$, $no=6$

For the active transport of statin acid anions by OATP transporter, rat hepatocytes, [Shitara 2013]: Equation 5

$$K_m = 1.79 \Delta G_{\text{desolvation}} + 2.67 \Delta G_{\text{lipophilicity}} - 7.57 \text{ Dipole Moment} - 0.61 \text{ Molecular Volume} + 471.67$$

Where $R^2 = 0.949$, $SEE = 7.35$, $SE(\Delta G_{\text{desolvation}}) = 1.22$, $SE(\Delta G_{\text{lipophilicity}}) = 1.96$, $SE(\text{Dipole Moment}) = 2.82$, $SE(\text{Molecular Volume}) = 0.19$, $F=4.61$, $\text{Significance}=0.33$, $n=6$

The correlation for the active transport of statin acids by OATP transporter, rat hepatocytes, [Shitara 2013] was very poor, suggesting that OATP transporters do not transport the acid species. Eq 4 and 5 are indicative of rat OATP transporters being active with both anion and lactone species.

Gemfibrozil and cyclosporin are known to be inhibitors of the OATP1B1 transporter, but not substrates, and to increase statin plasma concentrations. [6,41,76,77] The area under the curve fold ratio (AUCR) for gemfibrozil and cyclosporin are known [6, Kalliokoski 2009, table 4] and are analysed in equations 6a, 6b and 7 for the inhibition of eight statin acid and lactones by gemfibrozil and cyclosporin respectively. It is noted that there is some smaller contributions from CYP enzyme inhibition other than OATP1B1 alone, for example for atorvastatin, cerivastatin and simvastatin where some CYP3A4 inhibition is thought to be

involved for cyclosporin inhibition, and where some CYP2C8 inhibition is thought to be involved with gemfibrozil inhibition of OATP1B1. Hence the AUCR is not a sensitive or precise indicator of the inhibition of statin-transporter processes, but may shed light on systemic factors. These equations commonly show low dependence on molecular volume, and equations 6a and 6b) also show low dependence on $\Delta G_{\text{desolvation}}$. The correlation with the statin lactones are statistically stronger than for the acids, similar to the previous transporter correlation equations 4-5, implying a preference for lactone transport over the acid form of statins. There are ample literature references supporting a major role for lactones being involved in OATP transport along with the acid form. [6,26,38,41,76,77] The AUCR is inversely dependent on the clearance, and dependent on the dose administered and its bioavailability (see Table 1). For orally administered drugs, the bioavailability is dependent on many complex factors, so transporter inhibition effects can be confounded, which may explain the relatively small dependencies on lipophilicity and dipole moment. For example cyclosporin is an inhibitor of influx and efflux transporters such as OATP1B1, OATP1B3, MDR1, MDRP2, as well as CYP3A4. The AUCR values vary within the range of 1.5-4.4 and 3.5-20.0 for gemfibrozil and cyclosporin inhibition respectively. There was a very strong statistical relationship found for the AUCR values for cyclosporine inhibition as shown in equation 7, which may reflect cyclosporin's ability to inhibit both influx and efflux transporters. [6] The equations derived for cyclosporin inhibition of statin transporters are much more robust and mechanistically indicative. Mechanistically these equations are consistent with a facilitated transport channel for the statins which can be inhibited with gemfibrozil and cephalosporin, where the protein transporter channel size easily accommodates all the statins, the statins are essentially desolvated before entering the channel, and interaction between the protein and statins is predominantly a modest lipophilic (hydrophobic) interaction, coupled with a modest dipolar electrostatic interaction. These equations confirm the notion that OATP1B1 transport is a factor in statin hepatic uptake and systemic bioavailability.

For the OATP1B1 transporter inhibition by *gemfibrozil* of statin *acids*, Kalliokoski (2009): Equation 6a

$$\text{AUCR} = 0.12 \Delta G_{\text{desolvation}} + 0.38 \Delta G_{\text{lipophilicity}} + 0.11 \text{ Dipole Moment} + 0.01 \text{ Molecular Volume} + 1.04$$

Where $R^2 = 0.556$, $SEE = 1.12$, $SE(\Delta G_{\text{desolvation}}) = 0.15$, $SE(\Delta G_{\text{lipophilicity}}) = 0.21$, $SE(\text{Dipole Moment}) = 0.27$, $SE(\text{Molecular Volume}) = 0.01$, $F=1.00$, $\text{Significance}=0.54$, $no=8$

For the OATP1B1 transporter inhibition by *gemfibrozil* of statin *lactones* Kalliokoski (2009): Equation 6b

$$\text{AUCR} = -0.02 \Delta G_{\text{desolvation}} + 0.38 \Delta G_{\text{lipophilicity}} + 0.15 \text{ Dipole Moment} + 0.03 \text{ Molecular Volume} - 2.40$$

Where $R^2 = 0.866$, $SEE = 0.61$, $SE(\Delta G_{\text{desolvation}}) = 0.04$, $SE(\Delta G_{\text{lipophilicity}}) = 0.12$, $SE(\text{Dipole Moment}) = 0.08$, $SE(\text{Molecular Volume}) = 0.01$, $F=4.85$, $\text{Significance}=0.11$, $no=8$

It is noted that there was no correlation between statin acid anions and gemfibrozil AUCR, $F=0.26$, $\text{Significance}=0.89$ compared to those found for equations 6a and 6b.

For the OATP1B1 transporter inhibition by *cyclosporin* of 8 statin *acid anions*, Kalliokoski (2009): Equation 7

$$\text{AUCR} = 0.05 \Delta G_{\text{desolvation}} + 0.17 \Delta G_{\text{lipophilicity}} - 0.04 \text{ Dipole Moment} + 0.06 \text{ Molecular Volume} - 8.98$$

Where $R^2 = 0.994$, $SEE = 0.66$, $SE(\Delta G_{\text{desolvation}}) = 0.04$, $SE(\Delta G_{\text{lipophilicity}}) = 0.02$, $SE(\text{Dipole Moment}) = 0.09$, $SE(\text{Molecular Volume}) = 0.01$, $F=110.6$, $\text{Significance}=0.001$, $no=8$

The correlations with the lactones or acids were very poor. The AUCR correlations for gemfibrozil and cyclosporine inhibition show different patterns for the three possible statin species, but eq 7 is the most significant correlation, indicating desolvation, lipophilicity and molecular volume may dominate.

3.4 Models for statins as inhibitors of CYP enzymes:

CYP2C8 is one of the major drug-metabolizing CYP enzymes. It is important in the metabolism of many drugs including paclitaxel, rosiglitazone, pioglitazone, repaglinide and amodiaquine. In humans, gemfibrozil has markedly increased the plasma concentrations of the CYP2C8 substrate cerivastatin 5-6 times, and also increases the plasma levels of simvastatin acid and lovastatin acid but with little effect on the lactone forms. The *in vitro* inhibition (K_i) of simvastatin 7.1, simvastatin acid 41.1, lovastatin 8.4, lovastatin acid 48.9, fluvastatin 18.9, pravastatin >50, cerivastatin 31.7, rosuvastatin >50 and atorvastatin 15.9 μM on the CYP2C8 model reaction, paclitaxel 6 α -hydroxylation, in human liver microsomes has been studied. [78] The outer membrane of mitochondria have integral proteins called porins that allow molecules of up to 5000 Da to freely diffuse through, so the concentration of small molecules in the intermembrane space is the same as in the cytosol. The inner membrane contains proteins including those responsible for redox reactions of oxidative phosphorylation. A model equation to relate the K_i values to the free energy of desolvation, the dipole moment and the electron affinity (EA) is shown below as equation 8. The EA is a measure of the ease of reduction of a substrate, as occurs in the oxidation-reduction process occurring in cytochrome enzymes, eventually forming in this case, 6 α -hydroxypaclitaxel. Molecular volume is an irrelevant factor in this case as the statins are well under the 5000 Da limit. Equation 8 shows a strong relationship for the acids and lactones, particularly for the EA.

Simvastatin acid and lovastatin acid were ca. six times weaker inhibitors of CYP2C8 than their lactone counterparts, which is consistent with the higher EA for acids compared to lactones (see Table 2) which indicate that the acids are metabolised (hydroxylated) more readily than the lactone forms, which then act as stronger inhibitors when bound to CYP2C8. In terms of likely side effects caused by statin inhibition of CYP2C8, the K_i values are about 10-100 times the peak plasma concentrations of these statins at steady state and standard daily doses.[78]

For the statin (fluvastatin acid, atorvastatin acid, cerivastatin acid, lovastatin acid, simvastatin acid, lovastatin lactone, simvastatin lactone) inhibition of paclitaxel oxidation by CYP2C8, Tornio (2005): Equation 8

$$\mathbf{K_i = 6.17 \Delta G_{desolvation} + 5.51 \Delta G_{lipophilicity} - 5.12 \text{ Dipole Moment} + 16.60 \text{ Electron Affinity} - 16.0}$$

Where $R^2 = 0.993$, $SEE = 2.37$, $SE(\Delta G_{desolvation}) = 0.40$, $SE(\Delta G_{lipophilicity}) = 0.06$, $SE(\text{Dipole Moment}) = 0.73$, $SE(\text{Electron Affinity}) = 3.57$, $F=70.60$, $\text{Significance}=0.014$, $no=7$

The inhibitory effects of eight statin acids and lactones on CYP2C8, 2C9/10 and 2C19 and 3A4/5 metabolism and MDR1 transport activities with human liver microsomes and MDR1 over-expressing cell lines have been reported. Overall the acid forms showed little or no inhibitory effects, and the lactones showed small effects with the exception of CP3A4/5 activity, with IC_{50} values of atorvastatin 5.6, cerivastatin 8.1, fluvastatin 14.9, simvastatin 15.2, rosuvastatin 20.7 and lovastatin 24.1 μM . MDR1 mediated transport of digoxin was

inhibited only by the lactones, and the order was correlated with the same order for the inhibition of CYP3A4/5. [33] The paclitaxel 6 α -hydroxylation CYP2C8 IC₅₀ inhibition data is similar to the results obtained by Tornio [78], but Tornio has produced K_i data, intrinsic constants which are more accurate than IC₅₀ data (extrinsic constants are more dependent on experimental variables). The Sakaeda data analysis is shown in equation 9, which is far less significant than equation 8, but basically shows similar dependencies on the four dependent variables. *Of course these two data sets come from different experimental studies, but the agreement gives added confidence that the observed relationships are valid.* The substantial difference between equations 9 and 10 is the lesser inhibitory dependency on the lipophilicity and electron affinity of the statin lactones in equation 10. Equation 11 shows the IC₅₀ relationship for the 3-hydroxypaclitaxel CYP3A4/5 inhibition, which gives a similar result to equations 9 and 10, but is a marginally more statistically significant result. Equation 11 shows twice the dependency on EA for the CYP3A4 compared to the dependency on EA for the CYP2C8, which might suggest the former enzyme system may be a more potent metabolizer of statin lactones.

For the statin *acids* inhibition of paclitaxel oxidation by **CYP2C8**, Sakaeda (2006): Equation 9

$$\text{IC}_{50} = 12.07 \Delta G_{\text{desolvation}} + 10.72 \Delta G_{\text{lipophilicity}} - 8.73 \text{ Dipole Moment} + 71.65 \text{ Electron Affinity} - 115.4$$

Where R² = 0.916, SEE = 14.10, SE($\Delta G_{\text{desolvation}}$) = 2.77, SE($\Delta G_{\text{lipophilicity}}$) = 8.49, SE(Dipole Moment) = 4.05, SE(Electron Affinity) = 48.00, F=5.45, Significance=0.160, no=7

For the statin *lactones* (excluding rosuvastatin) inhibition of paclitaxel oxidation by **CYP2C8**, (Sakaeda (2006): Equation 10

$$\text{IC}_{50} = 6.9 \Delta G_{\text{desolvation}} + 10.9 \Delta G_{\text{lipophilicity}} - 10.0 \text{ Dipole Moment} + 51.6 \text{ Electron Affinity} - 110.8$$

Where R² = 0.871, SEE = 15.0, SE($\Delta G_{\text{desolvation}}$) = 2.9, SE($\Delta G_{\text{lipophilicity}}$) = 3.1, SE(Dipole Moment) = 6.4, SE(Electron Affinity) = 37.2, F=3.36, Significance=0.24, no=7

For the statin *lactones* (excluding rosuvastatin) inhibition of paclitaxel oxidation by **CYP3A4/5**, Sakaeda (2006): Equation 11

$$\text{IC}_{50} = 11.0 \Delta G_{\text{desolvation}} + 11.2 \Delta G_{\text{lipophilicity}} - 16.2 \text{ Dipole Moment} + 111.9 \text{ Electron Affinity} - 20.8$$

Where R² = 0.775, SEE = 23.4, SE($\Delta G_{\text{desolvation}}$) = 4.54, SE($\Delta G_{\text{lipophilicity}}$) = 4.83, SE(Dipole Moment) = 9.9, SE(Electron Affinity) = 57.89, F=1.73, Significance=0.39, no=7

The electrochemical peak reduction potentials E_p for the statin acids are related to the electron affinities EA. There are many examples of linear relationships between E_p and EA for reversible reactions. However the relationship does not strictly apply for irreversible reactions, which is the situation for the electrochemical studies of the statins (except pravastatin which can be reversible or irreversible depending on the conditions). Equation 9 describes the statin acids inhibition of paclitaxel oxidation by CYP2C8, which is a competitive inhibition of the complex redox processes involved in CYP2C8 metabolism. Since E_p describes the ease of redox reduction of the statin acids under electrochemical conditions, it may be instructive to see if there is any relationship between IC₅₀ values for paclitaxel oxidation by CYP2C8 and the known E_p values under conditions where the acid form is dominant, at pH 5 (equation 12). It can be seen that equations 9 and 12 are very similar, except that the significant dipole moment dependencies are reversed. If this observation is physically valid, and not a statistical aberration, this may be a result of the electrodic electrochemical environment being quite different from a purely aqueous

environment for the EA calculations. The dependency on desolvation energy is not statistically significant, and can be omitted with an improvement in precision. *Equations 9 and 12 do support the importance of electron transfer processes being important for CYP metabolism of statins*, and that the more easily obtained EA values can be used as substitutes for experimentally derived reduction potentials during drug screening evaluations.

For the statin *acids* inhibition of paclitaxel oxidation by **CYP2C8**, Sakaeda (2006): Equation 12

$$\text{IC}_{50} = -2.6 \Delta G_{\text{desolvation}} + 6.4 \Delta G_{\text{lipophilicity}} + 19.0 \text{ Dipole Moment} + 86.0 \text{ Peak Reduction Potential} + 13.8$$

Where $R^2 = 0.916$, $\text{SEE} = 11.9$, $\text{SE}(\Delta G_{\text{desolvation}}) = 3.5$, $\text{SE}(\Delta G_{\text{lipophilicity}}) = 2.4$, $\text{SE}(\text{Dipole Moment}) = 7.9$, $\text{SE}(\text{Peak Reduction Potential}) = 44.6$, $F=5.43$, $\text{Significance}=0.16$, $\text{no}=7$

Equations 8-12 above are suggestive that redox processes are involved in the inhibition of paclitaxel by CYP enzymes. The EA or peak reduction potential are strong indicators of the ease that electrons can be attached to the statin acids or lactones by the CYP enzymes. Since there is strong experimental evidence that the lactone forms of statins are more potent inhibitors of mitochondrial electron transfer processes than the acid forms, [32,33,48] an examination of the possible lactones species has been made.

It is known that the interconversion of statin acids to the lactones is pH dependent, with the lactone form dominant under acidic conditions, and basic conditions are required to completely transform lactones to the acid form. [8,9,14] Statin lactones have also been identified as strong inhibitors of mitochondrial complex III, [48] where proton gradients are involved. The intermediate species involved in acid conditions are the neutral lactone and the protonated lactone with a $-\text{C}(=\text{O})-\text{O}(\text{H}^+)$ moiety on the lactone ring. Hence it is likely that the active lactone species involved in redox processes between the statin lactones and the mitochondrial electron transfer chain could involve both the neutral and protonated lactone species. Comparisons of the calculated EAs of the neutral statin lactones and the protonated lactones show that the protonated lactones have higher EAs for all statins with the exception of rosuvastatin and cerivastatin, where the electron is not attached to the lactone ring (as indicated by the location of the highest occupied molecular orbital, HOMO), but elsewhere in the molecule. But for pravastatin, fluvastatin, atorvastatin, pitastatin, lovastatin and simvastatin, where the electron is attached to the lactone ring, the ratios of the EA for the protonated lactones divided by the EA for the neutral lactone, the ratios are 2.3, 1.4, 1.4, 1.0, 2.1 and 2.2 respectively. So these statin lactones are more susceptible to electron transfer from mitochondrial enzymes. This process has been confirmed by using a model lactone for the statins (by substituting a methyl group for the 'remaining' component part of the various statin lactone molecules) where the intermediate lactone ring structure shows an elongated $-\text{C}(=\text{O})-\text{O}(\text{H}^+)$ bond when an electron is attached to the protonated lactone model. This result is an example of the dissociative electron transfer mechanism. [79a,79b] The bond elongation is 2.7Å for the adiabatic EA (3.4eV) compared to 1.45Å for the vertical EA (1.7eV), which corresponds to a free energy change of -39.1 kcal/mol for the electron attachment induced bond elongation process in water. *These data are suggestive that the actual species involved in statin induced myopathy and other cytotoxic side effects may not be the statin lactones, but are likely to be the **protonated** statin lactones.* It is also suggested that the location of the electron attached to the statin lactones by mitochondrial enzymes may also be important differentiators of statin inhibition of metabolising enzymes. It is noted that pitastatin shows an unusually low EA ratio of 1.0 for the protonated lactone compared to the non-protonated lactone, which is consistent with the previous observation that pitastatin acid

and lactone are minimally metabolized by CYP enzymes, and so should have a lower risk of drug-drug interactions (DDI) than other statins whose disposition is dependent on CYP enzymes. A large post-marketing study conducted in more than 20,000 patients in Japan has demonstrated that the rate of DDIs with pitavastatin treatment may compare favourably with that observed with atorvastatin and rosuvastatin. [18,80] The ratios for fluvastatin 1.4 and atorvastatin 1.4 are considerably lower than those for pravastatin 2.3, lovastatin 2.1 and simvastatin 2.2, which could be suggestive of greater DDIs.

3.5 Inhibition of HMGCoA by statins

The inhibition of HMGCoA reductase by statins is well established. It is shown in eq 13 and 13a that this inhibition (in rat microsomes [86,87]) can be described by the four parameter model that also applies to transport and metabolic activity of statins.

Inhibition of rat microsomal HMGCoA reductase activity K_i (nM) by statin acid *anions*: rosuvastatin 12.0, pitavastatin 1.7, pravastatin 2.3, fluvastatin 0.3, atorvastatin 1.7, cerivastatin 1.3, lovastatin 0.6, simvastatin 0.12, mevastatin 1.4 (Corsini 1995) Rosuvastatin and mevastatin omitted as outliers

Equation 13

$$K_i = 0.002\Delta G_{\text{desolvation}} + 0.046\Delta G_{\text{lipophilicity}} + 0.224 \text{ Dipole Moment} + 0.033 \text{ Molecular Volume} - 17.79$$

Where $R^2 = 0.974$, $SEE = 0.229$, $SE(\Delta G_{\text{desolvation}}) = 0.016$, $SE(\Delta G_{\text{lipophilicity}}) = 0.008$, $SE(\text{Dipole Moment}) = 0.033$
 $SE(\text{Molecular Volume}) = 0.006$, $F=18.65$ Significance=0.051, $no=7$

Inhibition of rat microsomal HMGCoA reductase activity K_i (nM) by statin acid *anions*: rosuvastatin 12.0, pitavastatin 1.7, pravastatin 2.3, fluvastatin 0.3, atorvastatin 1.7, cerivastatin 1.3, lovastatin 0.6, simvastatin 0.12, mevastatin 1.4 (Corsini 1995) Rosuvastatin and mevastatin omitted as outliers

Equation 13a

$$K_i = 0.069 \Delta G_{\text{desolv,CDS}} + 0.048 \Delta G_{\text{lipophilicity}} + 0.247 \text{ Dipole Moment} + 0.031 \text{ Molecular Volume} - 17.68$$

Where $R^2 = 0.985$, $SEE = 0.176$, $SE(\Delta G_{\text{desolvation}}) = 0.057$, $SE(\Delta G_{\text{lipophilicity}}) = 0.006$, $SE(\text{Dipole Moment}) = 0.024$
 $SE(\text{Molecular Volume}) = 0.003$, $F=31.9$, Significance=0.030, $no=7$

Equations 13 and 13a are very similar and highly significant, and indicate the most sensitive binding variable is the dipole moment, which are high for the anionic species. The desolvation energy, lipophilicity and molecular volume of the statins are of about equal sensitivity. The data suggests that the non-polar solvation term $\Delta G_{\text{desolv,CDS}}$ best describes the desolvation or change in solvation as the ligands bind to the protein in the low dielectric environment of the protein pocket. Rosuvastatin is a very large outlier from the correlations, with mevastatin also a significant outlier. Rosuvastatin activity is sourced from a different study [87] than the other statin activity data from the one study. [86] Inclusion of mevastatin in eq 13 still gives a good correlation, R^2 0.877, F significance 0.100 but it is clear that eq 13 is a stronger correlation if mevastatin is treated as an outlier. Correlations of activity were tested against the statin acids, but the correlations were poor, indicating that the anionic species which are the dominant species at physiological pH, are the likely inhibitory species.

Conclusions

The neutral lactone and acid statin species are preferentially transported across the cell membrane and consequentially preferentially metabolised and cleared. The preferred cellular uptake of statin lactones has implications for cytotoxicity in muscle tissue and other side effects. The uptake mechanism is a combination of passive facilitated diffusion and active

permeation by OATP transporters. Equations 1-5 describe how passive diffusion facilitated uptakes rates, and binding affinity between statins and OATP transporters are related to the desolvation, lipophilicity, dipole moment and molecular volume components of a previously described membrane permeation model, [20] Equations 6-7 describe similar relationships between the cyclosporin and gemfibrozil competitive inhibition of statin-OATP1B1. Equations 8-12 describe the relationship between IC_{50} and the desolvation, lipophilicity, dipole moment and either the electron affinity, EA, or the electrochemical peak reduction potential for the statin inhibition of paclitaxel oxidation by CYP2C8. These equations suggest that electron affinity, a measure of reduction potential, is a useful indicator of potential drug-drug interactions (DDI). An examination of drugs known to cause DDI with statins show reduction potential and cellular uptake properties are useful predictors of DDI. These statistical models, supported by literature evidence, indicate the statin lactones play a previously unrecognized major role in statin therapeutics, and in side effects, cytotoxicity and DDI. It is likely that protonated statin lactones are the active species involved in electron transfer from the mitochondrial electron transfer processes that are a major cause of statin lactone induced myopathy. It has been demonstrated that the four parameter equation incorporating the independent variables desolvation, lipophilicity, dipole moment and molecular volume (or electron affinity) successfully correlates with passive and active transport processes (equations 1-7), the statin inhibition of CYP metabolic enzymes (equations 8-12), and the inhibition of HMGCoA reductase by statin anions (equation 13).

Material and methods

All calculations were carried out as previously described [61] using the Gaussian 09 package at the B3LYP/6-31G**(6d, 7f) level of theory with optimised geometries, as this level has been shown to give accurate electrostatic atomic charges, and was used to optimize the IEFPCM/SMD solvent model. With the 6-31G* basis set, the SMD model achieves mean unsigned errors of 0.6 - 1.0 kcal/mol in the solvation free energies of tested neutrals and mean unsigned errors of 4 kcal/mol on average for ions. [81] The 6-31G** basis set has been used to calculate absolute free energies of solvation and compare these data with experimental results for more than 500 neutral and charged compounds. The calculated values were in good agreement with experimental results across a wide range of compounds. [82,83]

Adding diffuse functions to the 6-31G** basis set (ie 6-31+**) had no significant effect on the solvation energies with a difference of ca 1% observed in solvents for the fluvastatin anion, which is within the literature error range for the IEFPCM/SMD solvent model. This is consistent with the finding [84] diffuse functions had a negligible effect on energy, geometry and charges for anions where conjugation or delocalisation of the negative charge was occurring.

The computation of the model lactone molecule was undertaken with a wB97XD/6-311++G(d,p) method since the wB97XD functional deals better with long range dispersion effects involved in the bond elongation accompanying electron attachment to the lactone model molecule. Free energy calculations were from thermochemical-frequency studies.

It is noted that high computational accuracy for each species in different environments is not the focus of this study, but comparative differences between various species is the aim of the study. The use of various literature values for passive diffusion rates, K_m , IC_{50} , AUCR etc to develop the multiple regression equations have much higher uncertainties than the calculated molecular properties. The statistical analyses include the multiple correlation coefficient R^2 ,

the F test of significance, standard errors for the estimates (SEE) and each of the variables SE($\Delta G_{\text{desolvation}}$), SE($\Delta G_{\text{lipophilicity}}$), SE(Dipole Moment), SE (Molecular Volume), SE(Electron Affinity), SE(Molecular Volume), SE(Peak Reduction Potential) as calculated from “t” distribution statistics. In some cases the coefficients of the variables were close to zero, and the coefficients were substantially smaller than the standard errors, indicating zero statistical significance of that variable. The peak reduction potentials were calculated by the method of Topol [85].

Table 1. Biophysical, metabolic and transport properties of statins

	Bio-avail. %	Half Life Hrs	Vol. of Distributn L	Log D	pK _a Acid	Active Metabolites	CYP Substrate	CYP 3A4/5 Binding ^C IC ₅₀ μ M	OATP Transport*	OATP Transport K _m μ M MM	Binding Energy ^A HMG-CoA Reduct. kcal/mol {IC ₅₀ ^A nM}	Plasma Protein Binding %
Atorvastatin	12	14	381	1.0-1.25	4.6	yes	3A4	5.6 Lactone 74.6 Acid	1B1* 1B3* 2B1* 2B1 ^E	0.6 ^F , 0.77*, 0.3 ^I 0.73*, 2.0 ^I 2.84* 0.2 ^E (heart)	-10.9 {1.7}	98
Fluvastatin	24 (9-50)	2.3	30	1.0-1.25	5.5	no	2C9	14.9 Lactone Nil Acid	1B1 1B3 2B2	1.4-3.5 ^G , 7.0 ^G 0.7 ^G (37.6 ^D OATP2)	-9.0 {0.3}	98
Lovastatin	5	3	na	3.91* 1.51#	4.3	yes	3A4	24.1 Lactone Nil Acid	1B1		{0.6}	95
Pitastatin	60	12	148	1.50	4.7	Minimal Lactone	2C8 2C9 Glu	67.2 Lactone 3A4/5 Nil Acid	1A2 1B1 1B3 2B1	3.0 ^H 3-4 ^H 3-4 ^H 1.2 ^H (3.9 ^B OATP2)		96
Pravastatin	18	1.3-2.7	35	-0.47	4.7	minimal	3A4 Sulf	73.7 Lactone Nil Acid	1B1 2B1	14-34 ^H 2 ^H (OATP2) 32.3 ^D	-9.7 {2.3}	50
Rosuvastatin	20	19	134	-0.25 to -0.5	4.6	yes minimal	2C9 Excr	20.7 Lactone Nil Acid	1A2 1B1 1B3, 2B1	3.0 ^H 9 ^H 10 ^H 2 ^H 7.3 ^B (OATP2)	-12.3	90
Simvastatin	<5	3	na	4.40* 1.80#	4.2	yes	3A4	15.2 Lactone 12.0 Acid	1B1		{0.12}	95
Cerivastatin	60	2.5	21	1.5-1.75	3.9	yes	3A4, 2C8	8.1 Lactone Nil Acid	1B1	4 ^H 4.3 ^B (OATP2)	-11.4	99

Footnotes:

Data from McFarland A, et al, Molecular Mechanisms Underlying the Effects of Statins in the Central Nervous System, Int.J.Molec.Sci., 2014,15,20607 and Schacter M, Chemical, pharmacokinetic and pharmacodynamic properties of statins: an update, Fundamental & Clinical Pharmacology 19 (2004) 117–125 and White MC, A review of the pharmacologic and pharmacokinetic aspects of rosuvastatin, J. Clin. Pharmacol., 2002, 42, 963.

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^A Binding energy (isothermal calorimetry) and IC₅₀ refer to binding of statins with HMG-CoA Reductase: Freire E, Do enthalpy and entropy distinguish first in class from best in class?, Drug Discovery Today, 2008, 13, 869.

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CYP Substrate: Glu = mostly glucuronidation, Sulf = mostly sulfuration, Excr = mostly excreted unchanged

* = lactone, # = acid.

Table 2. Solvation energies, lipophilicities, dipole moments, molecular volumes, ionization energies, electron affinities, reduction potentials of statins and interacting drugs

Statin	Solvation Energy Water - kcal/mol	Lipophilicity kcal/mol	Dipole Water D	Molecular Volume cm ³ /mol	IE eV Water	EA eV Water (Reduction Potential Volts pH 5) {Absolute Reduction Potl Calc}
Rosuvastatin Anion 90°	74.2	-37.3	38.6	331	5.8	1.7
Fluvastatin Anion 90°	85.1	-37.7	41.4	283	5.3	1.4
Atorvastatin 90° Anion	75.7	-40.7	35.0	374	4.6	1.0
Cerivastatin 90° Anion	94.1	-39.0	46.6	278	5.5	1.0
Pitavastatin 90° Anion	93.8	-40.5	45.7	299	5.5	1.8
Lovastatin Anion	87.8	-34.8	21.7	335	5.4	1.0
Pravastatin Anion	104.3	-40.8	37.5	356	5.4	0.8
Simvastatin Anion	84.5	-33.2	21.2	322	5.4	1.0
Rosuvastatin Acid 90°	32.7	-20.9	10.1	301	5.9	1.8 (1.18) {1.37}
Fluvastatin Acid 90°	23.7	-16.0	8.6	308	5.3	1.4 (0.64) {1.44}
Atorvastatin Acid 90°	26.7	-21.6	8.5	439	5.0	1.9 (0.92) {1.58}
Cerivastatin 90° Acid	37.2	-17.8	11.5	425	5.6	1.1 {1.32}
Pitavastatin Acid 90°	26.4	-18.1	6.2	335	4.8	1.5 (1.21) {1.52}
Lovastatin Acid	25.5	-15.0	5.1	340	5.4	1.1 (1.49) {1.63}
Pravastatin Acid	35.3	-17.0	9.4	298	5.4	0.8 (1.32) {1.38}
Simvastatin Acid	25.4	-14.6	5.1	378	5.4	1.0 (1.12) {1.70}
Rosuvastatin Lactone 90°	25.7	-16.8	7.7	316	6.2	4.6* {2.01}
Fluvastatin Lactone 90°	14.2	-16.4	1.5	304	5.0	0.7 {1.42}
Atorvastatin Lactone 90°	26.7	-23.3	5.5	355	5.0	0.8 {1.42}
Cerivastatin Lactone 90°	16.4	-15.9	10.1	342	5.8	1.7 {1.55}
Pitavastatin Lactone 90°	19.0	-17.5	9.1	300	5.6	1.9 {1.56}
Lovastatin Lactone	18.1	-14.5	4.0	292	5.4	1.0 {1.59}
Pravastatin Lactone	24.2	-15.6	4.8	295	5.4	0.6 {1.66}
Simvastatin Lactone	20.7	-16.3	4.2	349	5.1	0.5 {1.69}
Gemfibrozil	12.2	-10.4	3.9	198	6.0	0.6
Gemfibrozil -Ion	74.1	-32.2	27.6	200	5.4	0.3
Fenofibrate	10.5	-14.0	8.2	239	6.1	2.6
Fenofibric Acid	18.0	-13.4	7.5	269	6.7	2.4
Ezetinibe	16.1	-16.1	6.4	294	5.5	0.7
Warfarin	15.8	-13.7	3.6	185	5.9	1.7
Warfarin -Ion	58.2	-29.1	5.4	267	5.1	1.1
Verpamil	9.5	-12.4	8.2	416	5.2	0.6
Verpamil +Ion	62.4	-37.1	9.9	343	5.6	0.7
Niacin	12.9	-6.1	4.3	78	7.0	2.1
Niacin -Ion	70.5	-28.4	12.4	89	5.9	1.1
Mibefradil	20.0	-18.7	8.2	381	5.5	0.6
Mibefradil +Ion	72.7	-43.2	14.8	385	5.9	0.7
Midazolam	13.2	-12.1	4.2	246	5.9	2.0
Cyclosporin	54.0	-35.3	1.5	889	5.5	1.3
Digoxin	43.0	-23.1	18.3	515	1.4	1.4
Cimetidine	20.2	-15.9	6.9	205	5.9	2.0

Colchicine	15.7	-11.2	6.0	268	5.4	2.1
Ubiquinone	5.2	-26.3	3.5	575	5.7	3.5
Cysteine	16.1	-6.1	3.8	88	6.43	0.3
Glutathione	44.3	-13.4	7.4	159	6.35	1.2

Footnotes to Table 2:

Solvation energies are calculated using the SMD - Polarizable Continuum Model (IEFPCM), Unified Force Field, scaled van der Waals surface cavity. Solvation (free) energies are the differences between the energies of the optimised statin in the gas phase and in the particular solvent. A. V. Marenich, C. J. Cramer, and D. G. Truhlar, *J. Phys. Chem. B*, 2009, 113, 6378-96.

Lipophilicity is the solvation free energies in n-octane, essentially a repulsive energy.

IE, EA: Vertical ionization energy and electron affinity in eV. Calculated from by the SCF difference method for anionic form as $IE = E(M^-) - E(M)$ and $EA = E(M^-) - E(M^{\cdot-})$ at the optimised geometry of M^- , or for the neutral acid form as $IE = E(M) - E(M^+)$ and $EA = E(M) - E(M^{\cdot})$ at the optimised geometry of M .

The IE and EA for rosuvastatin lactone are clearly outliers from the range of values for other statins, as well as the acid and anion forms, and appears to be related to the lower atomic charges on the methanesulphonate group, which is unique to the approved statins.

[Reduction potential] are literature electrochemical values at ~pH 5. See text section 3.2.

{Absolute reduction potential} are calculated values as per Topol IA, et al, Experimental determination and calculations of redox potential descriptors of compounds directed against retroviral zinc fingers: Implications for rational drug design, *Protein Science*, 2001, 10, 1434.

Values in cm^3/mol are molecular volumes in water defined as the volume inside a contour of 0.001 electrons/Bohr³ density.

The angles 90° etc refer to the conformational angles between the 4-FC₆H₅- group and the relevant heterocyclic ring where applicable.

Table 3. Metabolic clearance rates for statins and major metabolic enzymes involved

	Metabolic Clearance Acid	Metabolic Clearance Lactone	Clearance Ratio Lact/Acid	CYP Enzymes Acid	CYP Enzymes Lactone
Rosuvastatin	1	71	71	-	CYP3A4
Fluvastatin	33	226	7	CYP2C9	CYP3A4
Atorvastatin	26	1892	73	CYP3A4	CYP3A4
Cerivastatin	21	622	30	CYP2C8 CYP3A4	CYP3A4
Pitavastatin	3	5	2	CYP2C9	CYP3A4
Simvastatin	28	1959	70	CYP3A4	CYP3A4

Footnotes:

Data from Fujino H, Kojima J, Ch 5, p 109, Focus on Statin Research: Drug metabolism and transporter properties of statins, in Focus on Statin Research, Wong BA, ed, Nova Science Publisher, 2006, NY.

Metabolic clearances, CL_{int} are $\mu\text{L}/\text{min}/\text{mg}$ protein.

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3.5 Inhibition of HMGCoA by statins

The inhibition of HMGCoA reductase by statins is well established. It is shown in eq 13 and 13a that this inhibition (in rat microsomes [86,87]) can be described by the four parameter model that also applies to transport and metabolic activity of statins.

Inhibition of rat microsomal HMGCoA reductase activity K_i (nM) by statin acid *anions*:
rosuvastatin 12.0, pitastatin 1.7, pravastatin 2.3, fluvastatin 0.3, atorvastatin 1.7, cerivastatin 1.3, lovastatin 0.6, simvastatin 0.12, mevastatin 1.4 (Corsini 1995) Rosuvastatin and mevastatin omitted as outliers
Equation 13

$$K_i = 0.002\Delta G_{\text{desolvation}} + 0.046\Delta G_{\text{lipophilicity}} + 0.224 \text{ Dipole Moment} + 0.033 \text{ Molecular Volume} - 17.79$$

Where $R^2 = 0.974$, $SEE = 0.229$, $SE(\Delta G_{\text{desolvation}}) = 0.016$, $SE(\Delta G_{\text{lipophilicity}}) = 0.008$, $SE(\text{Dipole Moment}) = 0.033$
 $SE(\text{Molecular Volume}) = 0.006$, $F=18.65$ Significance=0.051, no=7

Inhibition of rat microsomal HMGCoA reductase activity K_i (nM) by statin acid *anions*:
rosuvastatin 12.0, pitastatin 1.7, pravastatin 2.3, fluvastatin 0.3, atorvastatin 1.7, cerivastatin 1.3, lovastatin 0.6, simvastatin 0.12, mevastatin 1.4 (Corsini 1995) Rosuvastatin and mevastatin omitted as outliers
Equation 13a

$$K_i = 0.069 \Delta G_{\text{desolv,CDS}} + 0.048 \Delta G_{\text{lipophilicity}} + 0.247 \text{ Dipole Moment} + 0.031 \text{ Molecular Volume} - 17.68$$

Where $R^2 = 0.985$, $SEE = 0.176$, $SE(\Delta G_{\text{desolvation}}) = 0.057$, $SE(\Delta G_{\text{lipophilicity}}) = 0.006$, $SE(\text{Dipole Moment}) = 0.024$
 $SE(\text{Molecular Volume}) = 0.003$, $F=31.9$, Significance=0.030, no=7

Equations 13 and 13a are very similar and highly significant, and indicate the most sensitive binding variable is the dipole moment, which are high for the anionic species. The desolvation energy, lipophilicity and molecular volume of the statins are of about equal sensitivity. The data suggests that the non-polar solvation term $\Delta G_{\text{desolv,CDS}}$ best describes the desolvation or change in solvation as the ligands bind to the protein in the low dielectric environment of the protein pocket. Rosuvastatin is a very large outlier from the correlations, with mevastatin also a significant outlier. Rosuvastatin activity is sourced from a different study [87] than the other statin activity data from the one study. [86] Inclusion of mevastatin in eq 13 still gives a good correlation, R^2 0.877, F significance 0.100 but it is clear that eq 13

is a stronger correlation if mevastatin is treated as an outlier. Correlations of activity were tested against the statin acids, but the correlations were poor, indicating that the anionic species which are the dominant species at physiological pH, are the likely inhibitory species.