

ABSTRACT

The ability to identify and understand the potential adverse effects of new drugs early in the development process can help manage patient risk versus clinical benefit. Cerivastatin (CRV), simvastatin (SMV), lovastatin (LOV), atorvastatin (ATV), fluvastatin (FLV) and pravastatin (PRV) are in the statin class of cholesterol lowering drugs used in the treatment of hypercholesterolemia. All have been associated with hepatotoxicity and the skeletal muscle disorder rhabdomyolysis. CRV was recently withdrawn from United States markets because of reports of fatal rhabdomyolysis and high incidence of liver toxicity. It is believed that the mechanisms underlying these adverse effects are linked to energy depletion and apoptosis. The purpose of the present study was to screen the statin drugs in a battery of *in vitro* assays designed to evaluate cytotoxic, apoptotic, and oxidative stress potential. Rat hepatoma (H4IIE) cells were seeded into 96-well plates (10,000/well). Following a 48 hr equilibration period the cells were treated with compounds at concentrations of 0, 0.1, 1, 5, 10, 50, 100, and 300 μ M for 24 hr. Cytotoxicity was evaluated by measuring membrane leakage, mitochondrial function, and cell number. Oxidative stress was assessed by measuring total glutathione (GSH) and 8-isoprostane. Apoptosis was determined via caspase 3 activity. The hydrophobic statins (FLV, CRV, SMV, LOV, and ATV) produced a modest decrease in ATP, cell number, and mitochondrial function, but had pronounced effects on the reduction of total GSH pools and induction of caspase 3 activity. In contrast, the hydrophilic statin (PRV) had no measurable effects in this system. These data are consistent with published studies in which the mechanism(s) associated with statin toxicity are energy depletion and induction of apoptosis. Moreover, these data indicate that early screening with a cell-based system that evaluates several biochemical processes can provide important information on the relative safety of new drugs prior to entering animal studies or beginning clinical trials.

INTRODUCTION

Statin therapy can reduce blood cholesterol levels via the competitive inhibition of the rate limiting enzyme in cholesterol synthesis hydroxymethylglutaryl coenzyme A reductase (HMG-CoA). The safety profile of statins has been extensively evaluated and the major clinically significant adverse effects encountered with the use of these agents have been rare instances of marked hepatotoxicity, and myotoxicity (rhabdomyolysis) with acute renal failure secondary to myoglobinuria. The mechanism of statin induced toxicity been reported to involve cellular energy depletion, induction of apoptosis, and localized oxidative stress. Depending on the model, statins can increase or decrease oxidative stress.

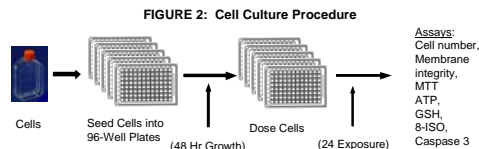
The manifestation of toxicity *in-vivo* is also related to pharmacokinetic and pharmacodynamic interactions dependent on chemical properties of the specific drug.

There is recent evidence to suggest that apoptosis related to statins could result in part from inhibition of geranylgeranylation of proteins involved in apoptotic signaling, but not by suppressing ubiquinone concentration nor via cholesterol synthesis inhibition in rat and human myotube cultures. (Johnson TE, et al. *Toxicol Appl Pharmacol*. 2004 Nov 1;200(3):237-50).

It has also been reported that the action of lipophilic statins can shift endothelial nitric oxide synthase localization towards intracellular domains, thereby producing intracellular oxidants. (Parker RA, et al. *Atherosclerosis*. 2003 Jul;169(1):19-29).

The mission of CeeTox, Inc. is to provide useable *in-vitro* toxicity screening early in the compound development process that can provide information on toxicity, structure-toxicity relationships, and the potential mechanisms of toxicity. This is achieved by analyzing the combined results of several biochemical assays chosen to monitor important cellular processes: membrane integrity, mitochondrial function, cell proliferation, oxidative stress, and apoptosis. This study examines several statin compounds in this system to include in the CeeTox *in-vitro* toxicity profiles database.

METHODS



Cell Culture

Cell culture procedure (Figure 2): Rat Hepatoma derived cells (H4IIE) were cultured for 48 hr in 96-well plates, then exposed to test compounds for 24 hours at concentrations of 0, 0.1, 1, 5, 10, 50, 100, and 300 μ M. Each exposure concentration was in septuplicate, and controls were included in each plate. Plates and cell media were subsequently analyzed using assays listed below.

Cytotoxicity Assays

Cell Number (Cell#)

Cell number was determined in a separate plate using a specific nucleic acid binding dye that fluoresces upon interaction with DNA and RNA. There is a direct correlation between intracellular RNA/DNA levels and cell number.

Membrane Leakage (Memb)

Acute cell death was determined by monitoring membrane leakage from cells using either ELISA or activity assays. The marker enzymes were specific for the tissue. Calculations were expressed as percent change relative to cell death as determined by complete cell lysis.

MTT

Cells remaining in each well were evaluated for their ability to reduce soluble 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT). The reduction of MTT has been linked to mitochondrial respiration and extramitochondrial reductase activity.

ATP Levels (ATP)

Cellular Adenosine triphosphate (ATP) was determined using a luciferase-based assay. This assay in combination with the MTT assay provides information on mitochondrial activity and energy status of the cell.

Oxidative Stress Assays

Glutathione (GSX)

Total glutathione was measured via a modification of the enzymatic recycling method.

8-Isoprostane (ISO)

8-Isoprostane was measured using standard procedures. 8-Isoprostane is produced by reaction of membrane phospholipids with oxygen radicals.

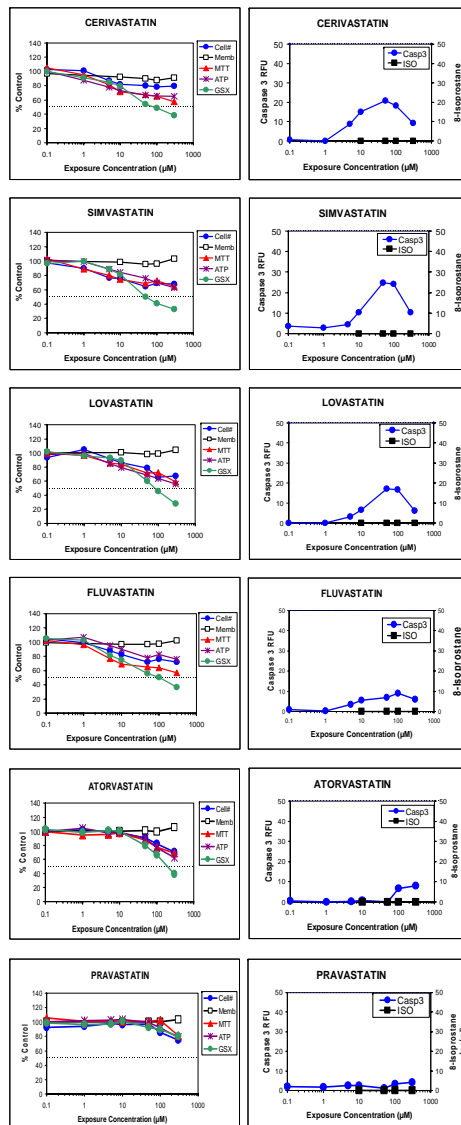
Apoptosis Assay

Caspase 3 (Casp3)

Caspase 3 activity (a late stage marker of apoptosis) was determined using a fluorescence-based assay with a caspase 3 specific substrate.

Solubility in the dosing medium was assessed with a Nephelometer. Interaction with P-glycoprotein (Pgp) was assayed at selected concentrations.

RESULTS



SUMMARY

The hydrophobic statins (CRV, SMV, LOV, FLV, and ATV) produced a modest decrease in ATP, cell number, and mitochondrial function, but caused marked reduction of total GSH pools and induction of caspase 3 activity. In contrast, the hydrophilic statin (PRV) had little effect in this system.

No membrane lipid peroxidation was observed at this time point with any of the compounds.

The compounds were soluble up to and including 300 μ M in this culture medium, and no substantial interaction with Pgp was observed (data not shown).

The Ctox values listed below are generated using a proprietary algorithm developed in-house to rank toxicity in this system.

The Octanol/H2O coefficient is a measure of lipophilicity at pH 7.0.

Compound	Ctox value (μ M)	Octanol/H2O Coefficient ⁽¹⁾
CERIVASTATIN	30	46.0
SIMVASTATIN	29	65.0
LOVASTATIN	34	16.0
FLUVASTATIN	40	22.0
ATORVASTATIN	100	15.0
PRAVASTATIN	100+	0.2

¹⁾ "Managing the Benefit With the Risk of Statin Therapy." Michael B. Bottorff MedScape CME

CONCLUSIONS

These results are consistent with current published hypotheses in which the mechanisms associated with statin toxicity are energy depletion, localized oxidative stress, and induction of apoptosis, with a relationship to the lipophilicity of the compounds.

Compared with the other statins, cerivastatin and simvastatin were implicated in a relatively higher number of clinically reported toxicity (Omar and Wilson. *The Annals of Pharmacotherapy*; Vol. 36, No. 2, pp. 288-295).

Statin with the least toxicity in this system are pravastatin and atorvastatin. Pravastatin has been associated with a lower incidence of myopathy (Hsu, et al. *The Annals of Pharmacotherapy*; Vol. 29, No. 7, pp. 743-759).

Clinical manifestation of statin toxicity is dependent on multiple additional factors such as the compound's pharmacokinetics and pharmacodynamics, dosage, drug-drug interaction and health of the patient.

Marked changes in energy status, localized oxidative stress, and apoptosis as measured in this system have been observed with other compounds that have been removed from the market due to hepatotoxicity such as troglitazone, nefazodone, and trovafloxacin.

These data indicate that early screening with a cell-based system that evaluates several biochemical processes can provide important information on the relative safety of new drugs prior to entering animal studies or beginning clinical trials. The CeeTox database includes a variety of characterized drugs and chemicals which can be compared to the toxicity profiles produced by new compounds.