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by

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Abstract

Lovastatin, a competitive inhibitor of the mevalonate pathway, is commonly prescribed for the treatment of hypercholesterolemia. More recently lovastatin has demonstrated an ability to induce tumour specific apoptosis however the mechanism by which lovastatin exerts this activity is not well characterized. End products of the mevalonate pathway are critical for various cellular functions including N-linked protein glycosylation, tRNA production, and the prenylation of a wide variety of cellular proteins among them many GTP-binding proteins which regulate cell proliferation, intracellular trafficking, and cell motility. Blockade of the rate-limiting step of the mevalonate pathway by HMG-CoA reductase inhibitors such as statins, results in decreased levels of mevalonate and its downstream products and thus may have significant influences on many critical cellular functions. This study demonstrates that the loss of key downstream mevalonate pathway metabolites leads to the activation of the cellular stress response known as the integrated stress response regulating lovastatin induced apoptosis in sensitive cell lines including head and neck squamous cell carcinoma (HNSCC), SCC25. The integrated stress response (ISR) is a signaling pathway, activated in response to a variety of different cellular stresses, that induces a common set of genes involved in cellular signaling leading to phosphorylation and inactivation of translation initiation factor, eIF2-α. The expression levels of several known mediators of the ISR were studied at both transcriptional and translational levels in response to lovastatin treatment. Lovastatin treatment induced eIF2-α phosphorylation and inhibited global protein translation. ATF4 expression was also induced, as well as increased ATF3 and CHOP expression (targets of ATF4) in sensitive cell lines only. In CHOP (-/-) murine embryonic fibroblasts (MEFs), lovastatin-induced apoptosis was attenuated indicating a role for CHOP in this response. Furthermore, the eIF2-α kinase, GCN2, mediates induction of ATF4 and lovastatin-induced apoptosis was also attenuated in GCN2 (-/-) MEFs. Our analysis revealed that lovastatin induced cytotoxicity directly correlates with induction of ISR markers ATF4, CHOP and ATF3 in sensitive cell lines. ATF3 is a transcription factor with dual oncogenic or tumour suppressor roles within the context of carcinogenesis and depending on the stressor can be induced during the ISR. Examining the role of ATF3 within the lovastatin induced cell death revealed that expression of GCN2 kinase and ATF4 are essential for its induction. In ATF3(-/-) MEFs, lovastatin-induced apoptosis was attenuated and induction of the pro-apoptotic transcription factor, CHOP, as well as its nuclear translocation was impaired. Consistently, depletion of *atf3* mRNA in two different cancer cell lines confers them partial protection against lovastatin induced apoptosis. Our analysis shows that GADD34, a target gene of CHOP required for de-phosphorylation of eIF2-α, was also induced by lovastatin treatment and abrogating the function of GADD34 by means of salubrinal, a specific inhibitor of GADD34 phosphatase activity, strongly enhanced cytotoxicity of low doses of lovastatin in MEFs and SCC25 cells and also strongly increased ATF3 expression levels. Therefore, combining mevalonate pathway inhibitors with agents that enhance the ISR represent a novel combinational therapeutic approach.

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List of Abbreviations

4E-BP1	eukaryotic initiation factor 4E binding protein 1
AARE	amino acid regulatory element
AKT	serine/threonine kinase
AML	acute meylogenous leukemia
asns	asparagine synthase
ATF	activating transcription factor
ATRA	all trans retinoic acid
BAEC	bovine aortic endothelial cells
Ca2+	calcium ion
CDK	cyclin dependent kinase
cDNA	complementary deoxyribonucleic acid
ChTL	chymotrypsin like
COX-2	cyclooxygenase-2
CREB	cyclic AMP response element binding protein
Ct	threshold cycle
CTD	carboxy terminal domain
CY3	cyanine3
cytC	cytochrome C
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Diaminoethanetetraacetic acid
EGFR	epidermal growth factor receptor
EIF2B	eukaryotic initiation factor 2B
eIF2-α	eukaryotic initiation factor 2 alpha
eIF4E	eukaryotic initiation factor 4E
ER	endoplasmic reticulum
ERAD	ER associated degradation machinery
ERO1α	ER oxidoreductin 1 alpha
ERSE	ER stress response element
FACS	fluorescent activated cell sorting
FBS	fetal bovine serum
FFA	free fatty acid
FPP	farnesyl pyrophosphate
GCN2	general control nonderepressible protein kinase 2
GDP	guanosine diphosphate
GFP	green fluorescent protein
GGPP	geranylgeranyl pyrophosphate
GTP	guanosine triphosphate
H2O2	hydrogen peroxide
HCl	hydrochloric acid
HisRs	histidyl-tRNA synthase
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HNSCC	head and neck squamous cell carcinoma
HRI	hemin-regulated inhibitor kinase
HSP70	heat shock protein, 70
ICAM1	intracellular adhesion molecule 1
IgG	immunoglobulin G
IB	inhibitory kappa B
IRE1	inositol requiring-1
ISR	integrated stress response
JNK	Jun N-terminal kinase
kg	kilogram
L-Arg	L-arginine

CHAPTER 1

1.1.Statins

1.1. a. Statins: a brief history

Statins are a class of drugs used to lower cholesterol in patients who suffer from or are at risk of cardiovascular or cerebrovascular disease. Their development was driven by the need for lipid lowering drugs with less harmful side effects compared to the agents that were available at the time (e.g. nicotinic acid, estrogen, cholestyramine, and dextrothyroxine) [1]. Lovastatin was the first to be FDA approved for the treatment of hyperlipidemia in 1987 and currently there are six others on the market [1]. Lovastatin, simvastatin, and pravastatin are derivatives of fungal metabolites while fluvastatin, atorvastatin, pitavastatin and rosuvastatin are synthetic analogues [1]. Comprehensive safety assessments indicate that statins are relatively safe and, as a result, they are extensively used, with simvastatin recently been made available over the counter in the United Kingdom [2].

Large randomized control trials were conducted to determine the efficacy of statin drugs for the treatment of cardiovascular disease as well as their safety [2]. In addition to showing the reduction in risk of heart disease and stroke as a result of the cholesterol lowering effects of statins, several unexpected observations were made through in depth analysis of this data which provided evidence that statin treatment may correlate with a reduction in risk of certain cancers; including, but not limited to, colorectal cancer, melanoma, prostate and breast cancer [2]. Further epidemiological studies of medical database records showed statin-associated reductions in overall cancer incidences averaging between 14-28% [2].

1.1.b Statins and the mevalonate pathway

Statins are small molecule, competitive inhibitors of 3-hydroxy-3-methyglutaryl coenzyme A (HMG-CoA) reductase, the rate limiting enzyme of the mevalonate pathway (Fig.1A). These drugs bind to the active site of HMG-CoA reductase inhibiting the conversion of HMG-CoA to mevalonate, a fatty-acid precursor to cholesterol. The structure of statins mimics HMG-CoA with additional hydrophobic side chains which increase the bulkiness of the molecule and are responsible for the stearic hindrance of substrate binding to the enzyme [3]. The side chains all have an open ring (acid) structure, which binds HMG-CoA reductase 1000 times more effectively than HMG-CoA, and a closed ring (lactone) structure, the inactive pro-drug, which is converted to the active drug by carboxyesterases in the liver and plasma [2], (Fig.1B).

Statins also inhibit synthesis of other downstream products of the mevalonate pathway that are important for cellular functions. These include dolichol, a carrier molecule in N-linked glycosylation of glycoproteins; ubiquinone, important for mitochondrial respiration; and the isoprenoid intermediates- farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). Isoprenoids are long, hydrophobic side groups that attach to various intercellular proteins, among them G-proteins and G-protein subunits of the Ras/Rho superfamily (Ras, Rho, Rab, Rac and Rap), facilitating their attachment to cellular membranes. Post-translational addition of FPP or GGPP (prenylation) is critical for the functionality of G-proteins and its disruption by statin treatment has significant effects on cell growth *in vitro* [4]. In healthy cells HMG-CoA reductase is controlled by the downstream products of the mevalonate pathway, for example, isoprenoids suppress HMG-CoA reductase by post-translational modification [5]. Tumour cells have been shown to be much more sensitive to this feedback inhibition where

isoprenoid metabolites are shown to cause G1 arrest and induction of apoptosis in certain tumour cells indicating that inhibition of HMG-CoA reductase is a potential targeted anti-cancer therapy approach [6].

1.1.c *Pleiotropic Effects of Statins*

In addition to lowering cholesterol, statin drugs have been shown to have other beneficial effects on various biological activities, including inflammation, angiogenesis and cell proliferation/apoptosis. These pleiotropic effects of statins can be either dependent or independent of HMG-CoA reductase inhibition of cholesterol synthesis. Statins have been shown to have anti-inflammatory and immunomodulatory effects *in vitro* and *in vivo* [7]. Lovastatin disrupts leukocyte adhesion by binding to lymphocyte-function-associated antigen 1(LFA-1) and reducing expression of intracellular adhesion molecule 1 (ICAM1) [8]. *In vivo* and *in vitro* studies also show statins modulate the differentiation of T-lymphocytes from pro- to anti-inflammatory [9]. Statins have been shown to both promote and inhibit angiogenesis and this appears to be dependent upon the dose, organ site, cell type or disease being studied. Statins are shown to protect several tissues from ischemia as well as reduce vasoconstriction [10]. In the mouse Lewis lung cancer model a high dose of cerivastatin resulted in a considerable (51%) decrease in tumour vascularisation [10]. Unrestrained cell proliferation and resistance to apoptosis are markers of carcinogenesis. The anti-proliferative activity of statins is well documented, for example, lovastatin is used experimentally to synchronize both normal and

Figure 1.A. Schematic of the mevalonate pathway. Summary of the key metabolites and downstream end products of the pathway. Statins are inhibitors of the rate limiting step of this pathway involving the conversion of HMG-CoA by HMG-CoA reductase.

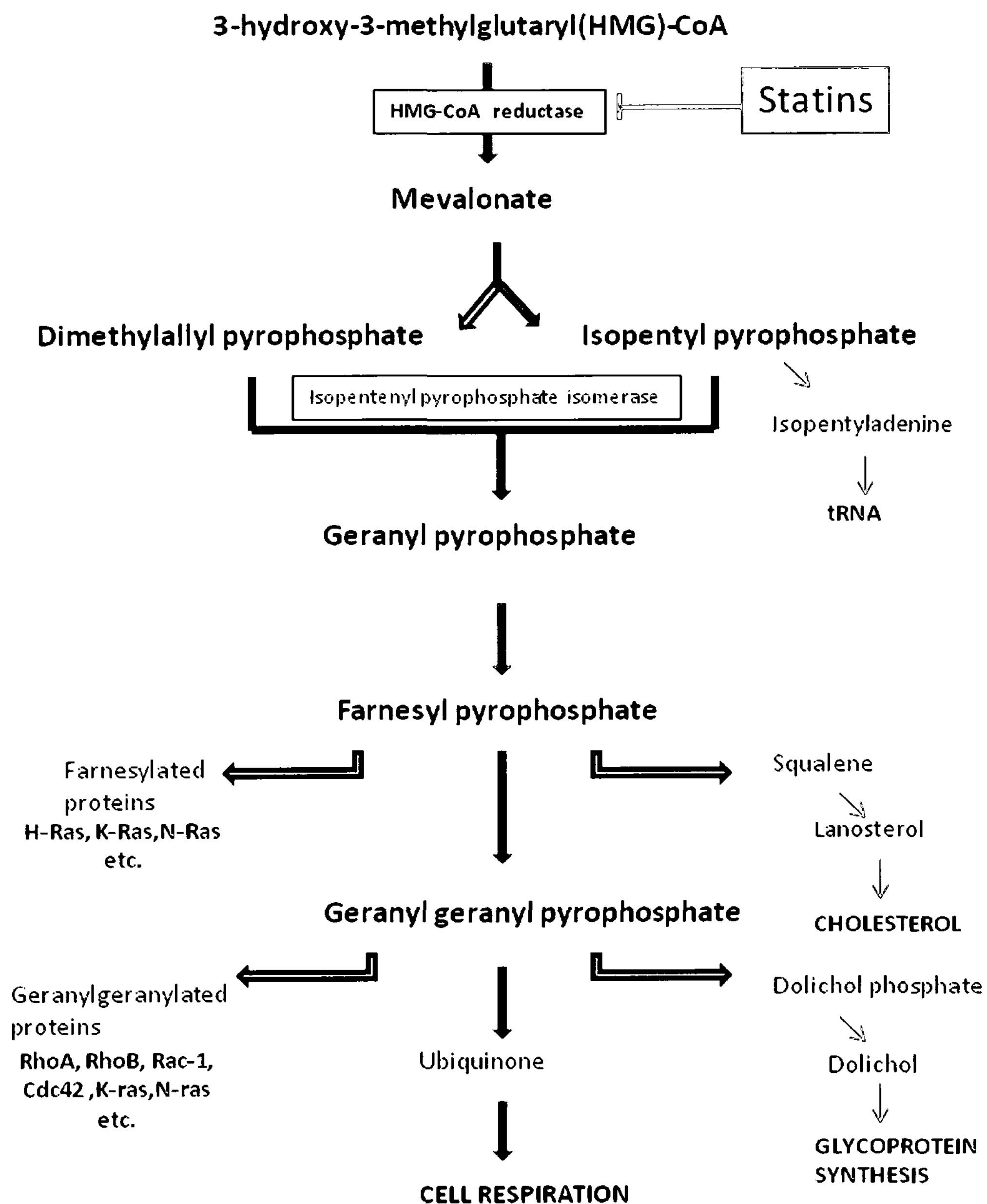
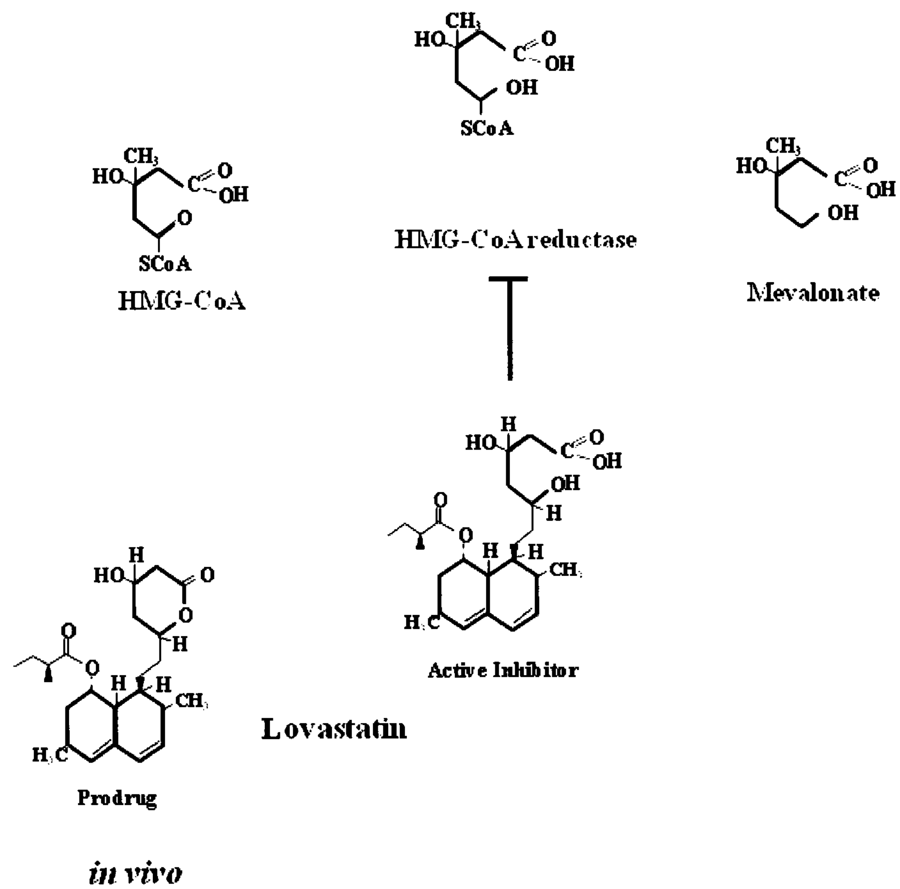

3-hydroxy-3-methylglutaryl(HMG)-CoA
HMG-CoA reductase
Statins
Mevalonate
Dimethylallyl pyrophosphate
Isopentyl pyrophosphate
Isopentenyl pyrophosphate isomerase
Isopentyladenine
tRNA
Geranyl pyrophosphate
Farnesyl pyrophosphate
Farnesylated proteins
H-Ras, K-Ras,N-Ras etc.
Squalene
Lanosterol
CHOLESTEROL
Geranyl geranyl pyrophosphate
Geranylgeranylated proteins
RhoA, RhoB, Rac-1, Cdc42 ,K-ras,N-ras etc.
Ubiquinone
CELL RESPIRATION
Dolichol phosphate
Dolichol
GLYCOPROTEIN SYNTHESIS

Figure 1.B. Structure of lovastatin. The prodrug form is converted to open ring activated form in the liver and plasma by carboxyesterases.


HMG-CoA
HMG-CoA reductase
Mevalonate
Active Inhibitor
Prodrug
Lovastatin
in vivo

tumour cells *in vitro* by blocking the progression of G1 to S and the cells can be rescued by addition of mevalonate [11]. This is mediated through p21 and/or p27 and may also involve inhibition of the proteasome although the exact mechanism of this controversial effect remains unknown [12].

1.1.d *Statins and Cancer*

There is extensive evidence that statins suppress growth in a variety of cells and this effect is stronger on malignant cells as opposed to non-malignant [6]. Statins appear to exert this effect on cell growth through interactions with the pathways associated with cell cycle, proliferation and differentiation. *In vitro* and *in vivo* studies show the ability of statins to induce apoptosis in a multitude of tumour cell lines, including leukemia, colorectal, prostate, breast, melanoma, and squamous cell carcinomas either alone or in combination with other chemotherapeutic agents and several of these studies are reviewed below.

Numerous reports have documented ability of statins to induce apoptosis in acute myelogenous leukemia (AML) cells [13, 14]. Exposure to 20μM lovastatin for 48hr strongly induced apoptosis in several AML cell lines and this effect was only abrogated by addition of GGPP while addition of FPP could only partially inhibit apoptosis and other downstream metabolites of the mevalonate pathway had no protective effect [15] . The pro-apoptotic effect of lovastatin is tumour specific as both primary and established tumour cells derived from AML patients are killed by lovastatin but myeloid progenitor cells isolated from healthy bone marrow or cord blood are resistant, even up to concentrations of 150 μM [16]. The mechanisms of statin induced apoptosis in leukemia are still under investigation but certain evidence points to the observation that high cholesterol levels in AML patients receiving chemotherapy correlate with a

resistance to certain chemotherapeutic agents thus inhibition of HMG-CoA reductase by statins may block this adaptation and in combination therapy statins could enhance the activity of chemotherapy agents in the clinic [17]. Statins have also been shown to trigger differentiation in leukemia cells [18]. Atorvastatin, fluvastatin and rosuvastatin induce apoptosis in NB4 acute promyelocytic leukemia (APL) cells and in variants resistant to all-*trans* retinoic acid (ATRA) statin treatment also promoted granulocytic differentiation reversing the resistance [14].

Studies have shown that colon cancer cells have higher HMG-CoA reductase activity and LDL receptor levels when compared to healthy mucosal cells [19] providing a possible link between cholesterol biosynthesis and colon cancer. Other possible mechanisms for statin effects on colon cancer involve inhibition of tumour cell growth through induction of cyclin-dependent kinase inhibitors as p21 is upregulated in HTC116 colon carcinoma cells following treatment with 10μM mevastatin [20] .

The observation that the growth of colon carcinoma cells could be inhibited by statin treatment *in vitro* led researchers to investigate whether statins could be used for cancer prevention *in vivo* in animal models of colon cancer. In mice with a mutation in the tumour suppressor adenomatous polyposis coli (*Apc*) gene, whose inactivation leads to the development of polyps (the precursor lesions in most colon carcinomas), treatment with atorvastatin suppressed intestinal polyp formation and this effect was augmented (83% less polyp formation than controls) when atorvastatin was used in combination with celecoxib, a COX-2 inhibitor shown to suppress colon cancer development [21].

A case-control study conducted by the Veterans Affairs Health Care System in the US showed a strong association between the use of statins and strong reduction (55%) in the risk of lung cancer incidence in patients using the drug for more than 6months [22] . *In vitro* statins

have been shown to strongly suppress growth of cell lines derived from both small cell lung carcinoma (SCLC) and non-SCLC lines [23].

In mice treated with tobacco-specific nitrosamine, 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NKK), tumour multiplicity was reduced by lovastatin exposure indicating possible tumour suppression at the early promotional stage [24]. Some of the proposed mechanisms for statin effects on lung carcinoma cells include inhibition of the function of the epidermal growth factor receptor (EGFR), high levels of which are observed in certain lung cancers [25]. In our lab we have previously shown that lovastatin inhibits EGF-induced EGFR auto-phosphorylation and when combined with gefitinib, an inhibitor of EGFR activation, the result is inhibition of ligand induced AKT activation and increased cytotoxicity [23]. The strong evidence of growth inhibition of tumour cells *in vitro* and *in vivo* indicates that statins are of potential use for the treatment and prevention of lung cancer.

Growth suppression and induction of apoptosis in breast cancer cells occurs following *in vitro* exposure to several different statins including fluvastatin, simvastatin, lovastatin and pravastatin [26]. *In vivo*, statins have been shown to inhibit growth of mammary carcinomas in the mouse ErbB2(+) breast cancer model [27] . The data from epidemiological meta-studies are unclear as to whether statins reduce the risk of breast cancer. Boudreau *et al.* [28] examined a cohort of 92,788 women studied from 1990 to 2004 and found that statin use was neither associated with an increase nor a decrease in the risk of developing breast cancer. Another study of early breast cancer survivors indicated a possible link between use of lipophilic statins and a lower risk of cancer recurrence post-diagnosis [29].

Several statins, including mevastatin, lovastatin, fluvastatin and simvastatin have been shown to cause G1 cell cycle arrest in human prostate cancer cell lines PC-3 and LNCaP and for

lovastatin this was observed with treatments corresponding to clinically relevant serum levels [30]. Simvastatin decreases AKT phosphorylation in LNCaP cells inducing apoptosis [31]. Li *et al.* [32] observed that prostate cancer cells contain more lipid rafts than other cell lines and apoptosis can be induced through agents that reduce cholesterol, including statins. *In vivo*, elevated cholesterol correlates with increased tumour growth, increased phosphorylation of AKT and resistance to apoptosis in xenograft tumours in SCID mice [31].

Epidemiological data provide conflicting information with regard to whether statin use correlates with a reduced incidence of prostate cancer. Several studies show treatment with statins is linked with reduced risk of prostate cancer [33]. Another epidemiological study in those individuals who were diagnosed with prostate cancer, statin use seems to reduce the risk of advanced, metastatic or fatal cancer incidences [34]. Further studies are required to establish a link between the *in vitro* observations of statin induced apoptosis on prostate cancer cells and the clinical observations to determine whether use of statins for prevention or treatment in prostate cancer is appropriate.

Many smaller studies have focused on the antitumour effects *in vitro* and *in vivo* in several other types of malignancies. Sensitivities to statin induced apoptosis *in vitro* are shown in thyroid cancer [35], glioma [36], medulloblastoma [37], and pancreatic cell lines [38]. Statins are shown to suppress the growth of multiple myeloma cells and to reverse multi-drug resistance in myeloma cells *in vitro* [39]. Inhibition of isoprenylation of Rho proteins by statins, which are important for metastasis of melanoma in humans, is shown to induce apoptosis and inhibit *in vivo* invasion of human melanoma cells [40].

Head and neck squamous cell carcinomas (HNSCCs) are rapidly proliferating solid tumours with very short doubling times (3-5 days) [41]. Radiation is the main therapeutic

modality, however failure of treatment to eradicate all of the rapidly growing tumour cells is common [42]. Our group has shown that HNSCC cell lines SCC9 and SCC25 are highly sensitive to lovastatin induced apoptosis and this effect is dependent upon the depletion of mevalonate [43]. As such there is strong interest in developing chemotherapeutic approaches using statins for the treatment of HNSCC.

1.1.e Clinical Trials

Numerous clinical trials were undertaken to assess the potential chemotherapeutic benefit of statins in light of the extensive effects on a variety of different tumour cells. However, the results have yet to demonstrate a clear chemotherapeutic benefit. In the first Phase I clinical trial involving 88 cancer patients, lovastatin was administered at a dose of 25mg/kg/day and this resulted serum concentrations of 0.1-3.9 μM, but while a minor response was seen by one patient with astrocytoma at the higher dose of 30-35 mg/kg/day, there were no significant antitumour effects seen on patients with other tumour types such as breast, prostate, and ovarian [44]. In another Phase I trial prolonged oral doses of lovastatin (7.5 mg/kg/day for 21days on a 28 days schedule) were given to patients with squamous cell cancers of the head and neck or cervix and there was documented stabilization of disease progression (23% patients had stable disease for more than 3 months) [45].

Clinical trials have also been conducted to determine the efficacy of statins when used in concert with standard therapeutic agents to augment the response. A Phase II trial evaluated the effect of pravastatin on 91 advanced hepatocellular carcinoma patients who were receiving oral doses of 200mg 5-fluorouracil (5-FU) and found that patients who received both agents had a median time of survival that was double the control (18 months vs. 9 months) [46]. A pilot

phase II trial involving six patients with drug resistant myeloma showed that addition of simvastatin along with bortezomib or bendamustine in follow up chemotherapy cycles resulted in a reduction of drug resistance [47].

The strong anti-proliferative activities of statins *in vitro* or in animal models has not yet been achieved in the clinic, however some of the more positive trial outcomes indicate that further studies are required to fully elucidate the molecular mechanisms of statin induced apoptosis in cancer cells. Determining what role the downstream products of the mevalonate pathway play in tumour sensitivity or identifying novel molecular targets of lovastatin will help to optimize the clinical utility of statins as anti-cancer agents either alone or as part of a combined therapy.

1.1.f Mechanisms

1.1.f.i Prenylation and disruption of Ras/Rab/Rho GTPases

Geranylgeranyl transferase and farnesyl transferase use GGPP and FPP, respectively, for post-translational modifications of a wide variety of cellular proteins [48, 49]. Among them members of the Ras superfamily of small GTPases which contains several subgroups, including Ras, Rab, and Rho families which function as molecular switches through binding of guanine nucleotides to mediate a complex array of cellular signaling pathways [50]. Ras proteins are membrane proteins that are responsible for the activation of signaling cascades in response to a variety of extracellular stimuli and have been shown to play a critical role in human oncogenesis [51]. Ras proteins interact with several effectors which regulate signaling networks controlling gene expression, cell proliferation, differentiation and apoptosis [50]. One of the most

characterized signaling pathway involves Ras activation through receptor tyrosine kinases, including the EGFR, leading to activation of prosurvival PI3K/AKT pathway.

Early studies focused on the role of FPP prenylation of Ras GTPases to explain statin induced apoptosis, however more recent studies indicate that GGPP prenylation of other G-proteins, including those of the Rab and Rho family, are critical for many of the pleiotropic effects of statins on apoptosis, angiogenesis, and inflammation. Add-back experiments involving GGPP or mevalonate generally protect cells from statin induced apoptosis whereas adding FPP does not or does only to a small degree [47]. About 0.5-1% of cellular proteins are geranylgeranylated but only a small subset of substrates have been identified [52].

The Rab GTPase family is a large subset of the Ras superfamily (61 members) and is involved in intracellular trafficking [53]. They are found both free in the cytosol and in active form associated with membrane-bound fractions [2]. Rab GTPases are involved in intracellular transport of vesicles and trafficking of proteins between organelles [54]. For example RAB1A, 2 and 25A are involved in ER-Golgi trafficking. Rab proteins also regulate the internalization and trafficking of amino acid transporters. Like Ras, Rab protein function has also been implicated in certain cancers. For example RAB25 promotes invasion in cancer cells and RAB1A is over-expressed in tumour cells derived from tongue squmaous cell carcinomas [55]. RAB5 has a crucial role in the regulating of growth-factor receptor internalization upon ligand binding and is expressed at higher levels in hepatocellular carcinoma and is implicated in lung adenocarcinoma as well [55]. RAB11 effector (RAB11-FLP1) can regulate the EGFR/AKT signaling pathway and overexpression of mutant RAB11A delayed EGFR recycling and inhibited proliferation of MCF10A cells [56].

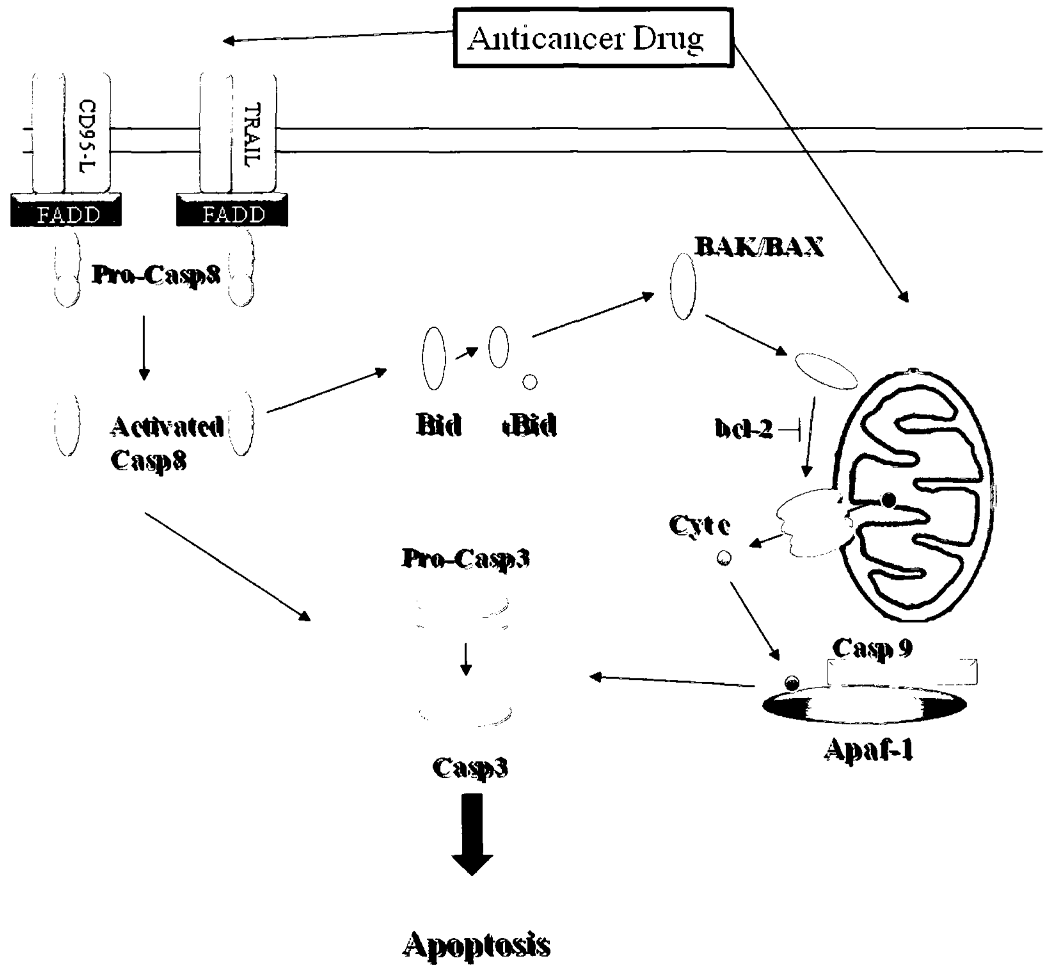
Rab proteins localize to specific intracellular membranes and as a result they require prenylation (specifically geranylgeranylation), that acts as a lipophilic membrane anchor, for their proper function. Lovastatin can inhibit transport of murine leukemia virus envelope protein to the Golgi apparatus, from the ER thus blocking its processing, presumably through disruption of Rab protein function [57]. In addition it is reported that lovastatin can also block Brefeldin A mediated retrograde Golgi-ER vesicles and this is likely due to the inactive non-isoprenylated form of RAB6 [58]. Interestingly, Brefeldin A blocks ER-Golgi trafficking of newly synthesized proteins a condition that can induce ER stress [59, 60]. As of yet no link has been demonstrated between lovastatin and induction of ER stress.

Rho GTPases are involved in the regulation of actin organization, cell cycle progression and gene expression through extracellular stimuli mediated signaling networks [61]. The most studied members of the Rho family include RHOA, which functions in actin stress fiber formation and focal adhesion; RAC1 which is involved in membrane organization; and CDC42 which also regulates organization of the actin cytoskeleton [50]. De-regulation of RAC1 is thought to play a role in tumour cell invasion [61]. Rho proteins also contribute to G1 cell cycle progression through inhibition of cyclin inhibitor p21 and induction of cyclin D1 expression [62]. Cerivistatin prevents prenylation of RHOA leading to G1/S cell cycle arrest mediated through AKT kinase pathways, NF-κβ inhibition and upregulation of CDK inhibitor p21 and this growth arrest was reversible through addition of GGPP but not FPP [63]. However the details of the mechanisms whereby disruption in functions of RHO/RAC/CDC42 by statins contributes to the anti-proliferative effects of statins remains poorly understood.

1.1.f.ii Statins and common apoptotic pathways

Initiation of apoptosis in tumour cells by cytotoxic agents can occur through stimulation of cellular death receptors which activate Caspase 8 and downstream targets (extrinsic) or within the cell at the level of the mitochondria mediated through BCL-2 modulation followed by the release of cytochrome C (intrinsic). Activation of either pathway results in the cleavage of pro-Caspase 3 initiating apoptosis (Fig.1.C). Statins induced apoptosis has been associated with both the death receptor and mitochondrial BCL-2 regulated apoptotic pathways. Up-regulation of BAX and BIM, down-regulation of BCL-2 [64], release of cytochrome C and cleavage of PARP in AML have been reported [65]. Expression of dominant active RHOA, a modulator of anti-apoptotic BCL-2, can inhibit lovastatin induced apoptosis by inhibiting down-regulation of BCL-2 [66]. In breast carcinoma the antitumour activity of statins has been linked to inhibition of the extracellular signal-regulated kinases (MEK/ERK) signaling pathway, reductions in activator protein-1 (AP-1) and NF-κB activation [27]. Lovastatin treatment in mouse mammary carcinoma activates p53-independent mitochondrial dependent apoptosis [67]. In prostatic epithelium, and in the prostate carcinoma derived cell line LNcaP lovastatin causes activation of Caspase 7 [68] while in leukemic HL-60 cells, protease activity of Caspase 3 has also been reported [65]. Interestingly, siRNA targeting of proapoptotic BH3-domain protein, BIM in sensitive cell lines, causes partial protection against lovastatin-induced apoptosis [64]. Lovastatin and specific geranylgeranylation transferase inhibitors can down-regulate anti-apoptotic MCL-1 in MM cells followed by loss of mitochondrial membrane potential [69]. Co-administration of cyclohexamide and actinomycin D with lovastatin revealed that protein synthesis and transcription machinery are required for lovastatin-induced apoptosis, respectively [70].

Figure 1.C. Mechanisms of apoptosis. Anticancer drugs can initiate apoptosis through intrinsic or extrinsic activation pathways. The extrinsic pathway involves stimulation of death receptors in the plasma membrane, such as TNF receptor superfamily member (CD95) or TNF-related apoptosis inducing ligand (TRAIL) resulting in receptor oligomerization and recruitment of Fas-associated death domain (FADD). This results in the binding and processing of pro-caspase 8 which initiates apoptosis by direct cleavage of downstream caspases. The intrinsic pathway involves stress signals which target the mitochondria directly causing cytochrome c release or is regulated by modulation of BCL-2. Cytochrome C release activates Caspase 3 and apoptosis through formation of the apoptosome complex which is made up of cytochrome C/APAF-1/Caspase 9. The extrinsic and intrinsic pathways can also converge through the BCL-2 family, BH3 domain containing protein, BID, whose cleavage by Caspase 8 can induce BAK/BAX. (Adapted from Fulda and Debatin, 2006 [71]).


Anticancer Drug
CD95-L
TRAIL
FADD
FADD
Pro-Casp8
Activated
Casp8
Bid
tBid
BAK/BAX
bcl-2
Cyt c
Pro-Casp3
Casp 9
Casp3
Apaf-1
Apoptosis

1.1.f.iii Lovastatin and ER Calcium

The endoplasmic reticulum (ER) is the major site of intracellular calcium storage. Under certain stress conditions calcium stores within the ER are released via inositol 1,4,5-triphosphate and ryanodine receptors [72]. Intracellular calcium stores are known to play a pivotal role in apoptosis. The role of calcium signaling in apoptosis became more complex with the finding that cytochrome C, following release from the mitochondria, goes to the ER and selectively binds to inositol trisphosphate receptors magnifying the release of calcium from the ER and consequently causes cell death [73]. Sustained ER calcium levels are essential for proper protein folding in this organelle. Drugs such as thapsigargin (TG), which disrupts the function of the ER calcium ATPase, and A23187, which depletes ER calcium, give rise to severe and rapid activation of ER stress also known as the unfolded protein response (UPR) [74].

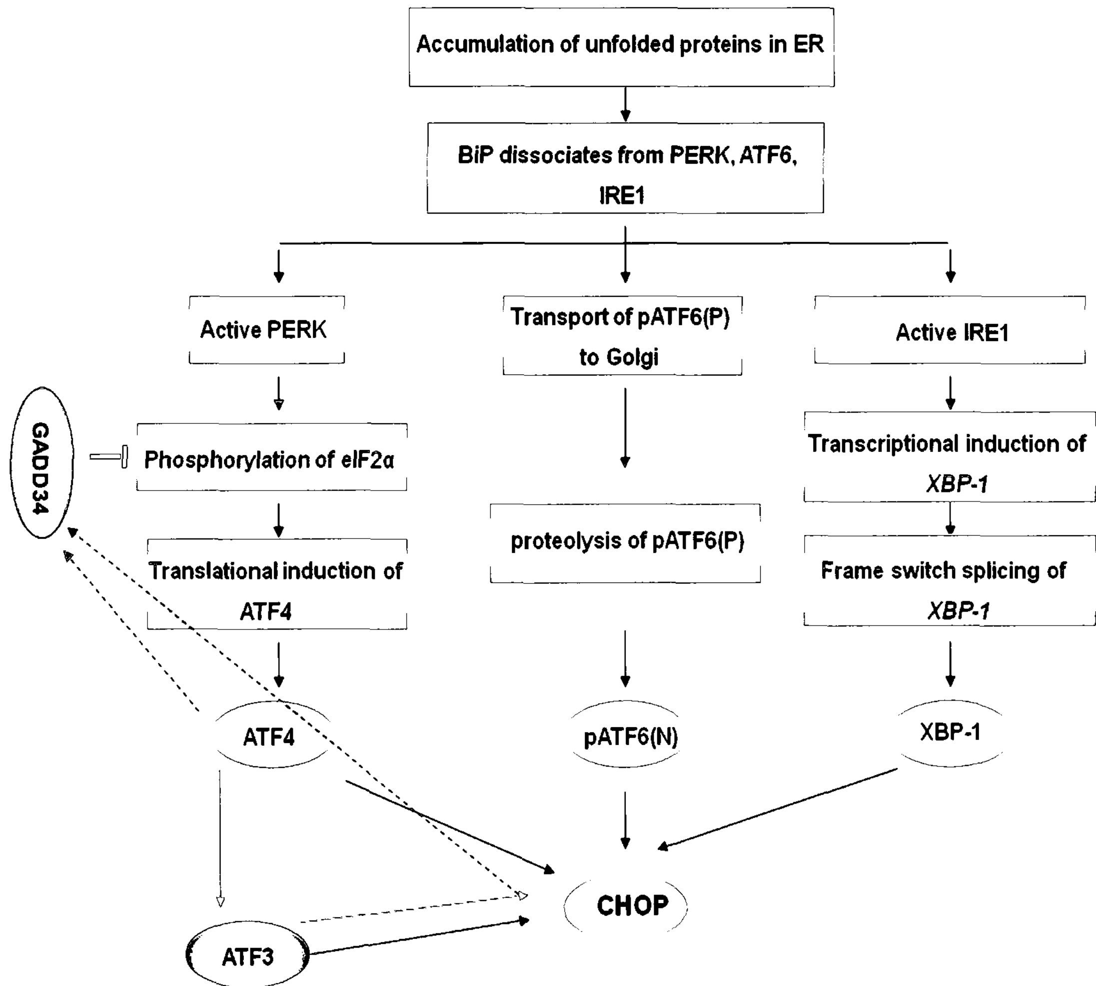
A recent study in bovine aortic endothelial cells (BAEC) showed an immediate increase in intracellular Ca^{2+} levels after treatment with several statins, including lovastatin [75]. Similar studies in L6 rat myoblasts [76], rat islet β-cells [77] and VSMCs (rat vascular smooth muscle cells) [78] also showed an increase in cytosolic [Ca2+] after treatment with simvastatin, in prodrug and acid-forms. Altogether these data suggest the involvement of ER stress in statin-induced apoptosis. Although several lines of evidence, such as intracellular calcium perturbations or inhibition of intracellular trafficking, suggest that statin treatment may induce the integrated stress response, so far there has been no independent examination of well-known markers of ER stress in response to statins, and in particular lovastatin.

1.2 UPR and ISR

1.2.a Pathway activation

Accumulation of misfolded proteins in the endoplasmic reticulum (ER) due to various stimuli or pathological conditions results in an evolutionary conserved response, the unfolded protein response (UPR), also known as ER stress. This response is activated to either alleviate the load of misfolded proteins by increasing the protein folding capacity of ER or alternatively to activate the signals to initiate apoptosis [79, 80]. Through upregulation of ER chaperones, such as BiP/GRP78 ER, shutting down global protein translation machinery, and decreasing the load of misfolded proteins by activating ER associated degradation machinery (ERAD), cellular stress may be overcome. The three main branches of the UPR are shown in Fig.1.D. BiP/GRP78, the ER chaperone, in the absence of stress condition binds to three ER lumen resident proteins, namely pancreatic eIF2- α PKR-endoplasmic reticulum-related kinase (PERK), activating transcription factor (ATF)-6 and inositol requiring 1-α (IRE1-α). Upon sensing unfolded proteins within the lumen of the ER, BiP dissociates from these proteins leading to their activation or alternatively, these proteins can be directly activated by unfolded proteins [79]. PERK and IRE1--α are activated through dimerization and autophosphorylation, while ATF6 is activated through transfer to Golgi apparatus followed by cleavage by two Golgi-resident type I proteases, S1P/S2P, which also process the SREBP family of transcription factors , following which it moves to the nucleus to bind to its target genes [79]. IRE1-α is an endoribonuclease that is responsible for the splicing of an intron from the mRNA of the transcription factor XBP-1 [81]. Only the spliced form of XBP-1 is an active transcription factor that migrates to the nucleus to activate its target gene(s). The UPR can be activated by disruption of protein glycosylation (through glucose deprivation or treatment of cells with drugs that directly inhibit glycosylation such as tunicamycin), perturbations in calcium homeostasis of the ER (TG), disruption of

Figure 1.D. Schematic of the unfolded protein response (UPR). Three branches of a conventional ER stress or UPR are depicted. Following detection of mis-folded proteins within the lumen of the ER, PERK alone or in combination with IRE1-α and ATF6 branches, can be activated to ameliorate cellular stress or alternatively promote apoptosis by inducing the expression of CHOP.



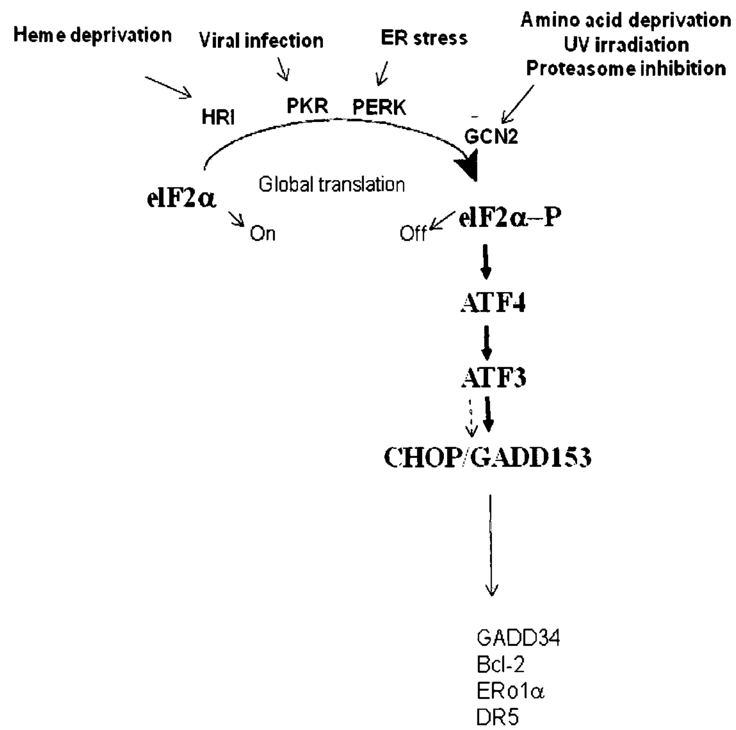
Accumulation of unfolded proteins in ER
BiP dissociates from PERK, ATF6, IRE1
Active PERK
Transport of pATF6(P) to Golgi
Active IRE1
GADD34
Phosphorylation of eIF2α
Transcriptional induction of XBP-1
proteolysis of pATF6(P)
Translational induction of ATF4
Frame switch splicing of XBP-1
ATF4
pATF6(N)
XBP-1
CHOP
ATF3

disulfide bond formation (dithiothreitol [DTT]), disrupting intracellular trafficking (brefeldin A) and hypoxia [82]. The branch of the UPR involving phosphorylation of the translation initiator, eIF2-α and upregulation of transcription factor, activating transcription factor-4 (ATF4), with their downstream targets, is in common with other stress induced signaling pathways caused by amino acid deprivation, heme deficiency and viral infection and taken together make up the integrated stress response (ISR) (Fig. 1E).

The phosphorylation of eIF2-α, in higher eukaryotes is regulated by four protein kinases; the double-stranded (ds) RNA-activated protein kinase (PKR), PERK, the general control nonderepressible-2 kinase (GCN2) and the hemin-regulated inhibitor kinase (HRI). eIF2-α is the sole known substrate of these kinases, with the exception of PERK (discussed below). Each of these kinases is activated by distinct stress conditions that lead to activation of ISR. PKR is activated by double-stranded RNA or viral infection and could represent an evolutionary pathway linking cellular suicide due to ER stress following viral infection; as a means of controlling viral replication and spread of infected cells [79]. HRI is activated by heme deficiency in erythroid cells or arsenite treatment in MEFs [83, 84]. Both PKR and HRI are also modulated by nitric oxide stresses in breast cancer cells [85]. For all four kinases of eIF2-α, autophosphorylation of certain conserved Thr residues is essential for their catalytic activities [86].

PERK is an ER-localized, type-I transmembrane protein with four domains, a signal peptide region, transmembrane domain, kinase domain, and amino terminal region. PERK homologues have been identified in both vertebrates and invertebrates. The amino terminal of PERK, which inserts into the lumen of the ER shares homology with the comparable region of

Figure 1.E. Schematic of the integrated stress response (ISR). Upon activation of any of the four eIF2α kinases by various cellular stresses, global protein translation machinery shuts down and selective translational induction of ATF4 results in the upregulation of downstream ISR-directed genes.



Adapted and modified from Wek et al., 2006

ER responsive gene, IRE1-α [87]. In non-stress conditions, the amino terminal regulatory domain of PERK interacts with ER chaperones, BiP (GRP78) and GRP98 maintaining the kinase domain in an inactive conformation [88]. Under stress conditions the luminal domain senses the accumulation of and directly binds to misfolded proteins within the ER leading to oligomerization and activation of PERK or alternatively, transformation to active form occurs through dissociation from BiP/GRP78, the ER chaperone, promoting dimerization and autophosphorylation [89]. Deletion of PERK has also been linked to disruption of intracellular calcium signaling through an as yet unknown mechanism [90].

In a global gene expression assay of PERK null MEFs, it was observed that amongst three ER stress inducers only TG, and to a lesser extent DTT, could trigger the PERK-mediated activation of JNK (c-Jun N-terminal kinase) and p38 MAPK pathways which are also associated with intracellular calcium level perturbations, however this is not seen with tunicamycin [91]. This suggests the specificity of PERK in sensing and induction of ER stress, such that it is able to activate different downstream signaling pathways depending on the initial stimulus [91].

It is noteworthy that PERK is the only eIF2-α kinase that has more than one known substrate. Cullinan and Diehl identified NRF2 as a novel substrate for PERK, which is involved in regulation of redox homeostasis [92], thereby providing a prosurvival function for PERK. Additional prosurvival activities of PERK activity include the activation of NF-κβ, an inducer of anti-apoptotic BCL-2[93].

Although during conventional ER stress, activation of all three branches of the UPR, including PERK, is seen there are examples of selective induction of the PERK branch only, depending on both type and severity of stress. For example the proteasome inhibitor, PS-341 (bortezomib) activates PERK and downstream targets, ATF4 and CHOP while simultaneously

inhibiting of IRE1-α activity and its target gene *XBP-1* inducing apoptosis in multiple myeloma (MM) cells [94].

Several studies have linked activation of PERK with increased tumour growth and survival under hypoxic conditions. Overexpression of PERK is associated with increased levels of phosphorylation of eIF2-α [95]. In addition, malignant murine cells with impaired PERK function, or with mutant, non-phosphorylatable eIF2-α, formed much smaller tumours with more apoptotic cells in hypoxic regions of the tumour compared to the wild type in xenograft models, suggesting ISR plays a vital role in tumourigenesis [96] . A K-Ras-transformed, PERK null mouse model also showed reduced tumour growth and angiogenesis [97]. Altogether, these data underline the importance of PERK and hence ISR, in tumour development and its potential as a candidate therapeutic target.

GCN2, a constitutive dimer, was first identified in yeast and is the most conserved eIF2-α kinase, present in all eukaryotes [98]. GCN2 is activated by sensing uncharged tRNAs that accumulate in cells due to amino acid starvation [99, 100]. The structure of GCN2 consists of five main domains; an amino-terminal charged region, a pseudo-kinase domain lacking catalytic residues, a kinase domain for phosphorylation of eIF2-α, a domain resembling histidyl-tRNA synthase (HisRs) and a carboxy terminal domain (CTD) that dimerizes in order to enhance tRNA binding and facilitate ribosome binding [101]. De-acylated tRNAs bind to both the HisRs and carboxy terminal domains with higher affinity compared to acylated forms [102]. Upon tRNA binding, the CTD and HisRs domains are released from the kinase domain, enabling recognition of its substrate (eIF2-α) [102]. The majority of the research about the function and activation mechanisms of GCN2 is done in yeast systems; therefore many of these findings have not yet been reported or observed in mammalian systems. To date, several of the identified inducers of

GCN2 in yeast are glucose, purine limitation, high salinity, rapamycin and MMS (methylmethanesulfonate) while in human cells amino acid starvation, UV irradiation and the proteasome inhibitor, MG132 have been shown to activate GCN2 kinase activity [103-106].

GCN2 is highly expressed in the brains of mammals [104, 107] and flies [108] . Studies have shown that the anterior piriform cortex (APC) of rat brains are sensitive to a lack of essential amino acid and this can cause phosphorylation of eIF2-α by the canonical mechanism of GCN2 activation [109] , which occurs through sensing of uncharged tRNA in APC neurons [110]. Additional roles for GCN2 in neuronal activity are reported in a GCN2 knockout mouse model, which showed long term memory potentiation impairment [111]. Interestingly, GCN2 null mice do not exhibit any neural impairment while they are fed on a normal diet [86]. However, when GCN2 null pregnant mice are fed an amino-acid deficient diet, their newborns show moderate developmental disadvantages compared to those born of wild-type dams [86, 112]. Under similar diet conditions, adult GCN2 null mutants show an inability to conserve their muscle mass [113] . Altogether, this data suggests that GCN2 might have compensatory physiological mechanisms to cope with amino acid deficiencies. GCN2 has also been shown to play a role in immune response, as kinase activation and induction of the ISR is observed in activated T-cells expressing tryptophan degrading enzyme indolamine 2,3-dioxygenase (IDO), which is an enzyme important for modulation of adjacent T-cells and is also believed to play a role in tumour anergy [114].

It is suggested that induction of ISR by MG132 is GCN2 dependent in MEFs [115], although this view has been recently challenged with a new report that suggests the major kinase involved in phosphorylation of eIF2-α is HRI rather than GCN2 [116]. More intriguingly, a recent report suggests that treatment of hepatocytes with MG132 at low concentrations activates

PERK and at higher concentrations (50μM) involves GCN2 kinase activity for phosphorylation of eIF2-α, independent of HRI [117] . Some of these discrepancies can be due to cell type specific effects. Another report suggests that GCN2 and PERK-regulated genes in mouse liver are differentially regulated at both transcriptional and translational levels [118]. This could be the result of activation of other arms of the ER stress pathway, following the initial stress stimulation.

UV irradiation is known to lead to activation of NF-κB in order to repair DNA damage and control cellular proliferation [119, 120]. A recent study demonstrated that UV-B and –C irradiation can lead to activation of NF-κB in a GCN2-dependent manner [103]. Reductions in the rate of protein synthesis, the result of phosphorylation of eIF2-α by GCN2, combined with turnover of IκBα, the inhibitor of NF-κB, leads to reduced levels of IκBα and consequently activation of NF-κB [93, 106]. Remarkably, GCN2 null MEFs showed increased apoptosis, following UV irradiation presumably due to regulation of NF-κB by GCN2. Moreover, there were no detectable levels of downstream ATF4 or pro-apoptotic transcription factor, CHOP, likely due to low abundance of *Atf4* mRNA, coupled to UV irradiation, suggesting that under certain stress conditions phosphorylation of eIF2-α is not sufficient to elicit the expression of ATF4 and its target genes [121]. As mentioned earlier the only known mechanism of GCN2 activation is the presence of uncharged tRNAs. What is still unclear is the nature of the cellular sensor of UV irradiation responsible for GCN2 activation. It is possible that UV, by damaging nucleic acids, including tRNAs, produces aberrant structures that interfere with their ability to charge with amino acid through acylation. An alternative possibility is the formation of a covalent crosslink between GCN2 and aminoacylated tRNAs by UV, which can activate GCN2 [103]. However, it seems under prolonged UV irradiation PERK also participates in

phosphorylation of eIF2-α [122]. Consistent with this latter study, a recent report proposed a model that induced nitric oxide synthase (NOS), during UVB-irradiation, generates $NO^{\cdot}$ from L-Arg, causing activation of GCN2 and then the uncoupled NOS generates O_2 leading to oxidative-stress and activation of PERK [123] .

1.2.b Downstream effects of ISR Activation

1.2.b.i Phosphorylation of eIF2-α and translation initiation

eIF2 is the essential component of translation initiation, and consists of three subunits α, β and γ. It binds to the initiator methionyl-tRNA (Met-$tRNA_i^{Met}$), in a GTP-dependent manner, through its γ site that associates with the small 40s ribosome. eIF2-α cycles between GTP bound and GDP bound states, recycling the protein between active and inactive states, respectively. The conversion is catalyzed by the guanine nucleotide exchange factor, eIF2-B, in order to reconstitute a functional ternary complex (TC) for another round of translation initiation. However, phosphorylation of eIF2-α converts the GDP bound kinase into a competitive inhibitor of eIF2B preventing the conversion of GDP to GTP bound eIF2B [86, 124]. Because most cells contain more eIF2 than eIF2B, phosphorylation of only a fraction of eIF2-α is sufficient to result in eIF2B sequestration into inactive eIF2-eIF2B complexes leading to inhibition of protein synthesis[86, 124].

Most organisms have several eIF2-α kinases and the conserved residue, Ser-51, is the common site for phosphorylation. Phosphorylation of eIF2-α at Ser-51 by the four kinases, PKR, HRI, PERK and GCN2, leads to general protein translation inhibition. Conversely, constitutive overexpression of non-phosphorylatable eIF2-α-S51A in MEFs leads to a three-fold higher rate

of protein translation [125]. A downstream consequence of phosphorylation of eIF2-α also includes the selective translational and transcriptional induction of signaling pathways that make up the ISR. Protein synthesis accounts for about 40% of total cellular energy and oxygen consumption of the cell thus shut down of global synthesis and selective induction of "stress genes" can help cells to allocate their energy resources to alleviate the stress condition [126].

Control of mRNA translation through translation initiation is a common mechanism of gene regulation in eukaryotes. Phosphorylation of eIF2-α inhibits general translation machinery from translation initiation, however it also induces translational upregulation of a certain mRNA, including activating transcription factor, *ATF4*, that contain upstream open reading frames (uORFs) [86, 127] . Interestingly, a recent study in mouse liver showed that about 2.5% of mRNAs are subject to translational induction following stress conditions that activate GCN2 and PERK [118]. The gene specific control of translation is not limited to *ATF4* only, other genes like arginine/lysine transporter (*CAT-1*) or CCAAT/enhancer-binding protein (*C/EBP*α) are subject to such regulations as well [128, 129]. It is noteworthy that phosphorylation of eukaryotic elongation factor 2 kinase (eEF2), similar to eIF2-α, suppresses global translation and stimulates the translation of specific transcripts that appear to be important for certain neurological activities [130]. There is also the suggestion that inhibition of eEF2 kinase by amino acids may prevent activation of GCN2, through the removal of deacylated forms of tRNA from ribosome P sites [124, 130].

It is proposed at least in terms of physiological regulation of neuronal activity by p-eIF2-α, weak levels of phosphorylation is sufficient to trigger induction of ATF4 and not affect general translation [127]. Thus, under these circumstances it appears that the induction of select mRNA and translation of a select subset of these mRNAs is more important than inhibition of

global protein synthesis. However, under extreme conditions, the overall balance between global protein inhibition and select translation upregulation may vary [86].

Upon induction of the ISR, translation inhibition by phosphorylation of eIF2-α contributes to the loss of cyclin D1, one of the regulatory D type cyclins necessary for G1/S transition, provoking G_1 arrest as a consequence of ISR induction [131]. Cell cycle arrest allow cells to shift their energy resources toward alleviating the stress condition and to restore homeostasis rather than permitting proliferation, which is a high energy consuming phenomena. The phosphorylation of eIF2-α and repression of cyclin D1 are mediated by both PERK and GCN2 during ER stress induced by tunicamycin [132]. However when ISR is induced by UV, GCN2 has the primary role for phosphorylation of eIF2-α repression [103]. It is noteworthy that extended treatment of PERK null MEFs with ER stressors can lead to robust phosphorylation of eIF2- α, however this is not seen in double knockouts of GCN2/PERK MEFs [106], suggesting physiological redundancies between these two kinases. Moreover, overexpression of a non-phosphorylatable form of eIF2-α in mouse NIH-3T3 is transforming and promotes tumourigenesis in xenograft mouse model [133]. This is in agreement with the anti-proliferative effect of phosphorylation of eIF2-α by promoting G1 cell cycle arrest [131].

1.2.b.ii Translation control in cancer

The requirement of cells to double their protein mass prior to mitosis underlines the importance of translation in the growth and proliferation of cells, including cancer cells. Reducing protein synthesis by half is sufficient to enter cells into quiescent G_0 arrest until normal protein synthesis is resumed [134]. Larger nucleoli, indicators of higher rates of rRNA synthesis and ribosomal assembly, is used as a pathological marker of cancer malignancy [135].

These irregularities in ribosome production likely involve the aberrant translation rates of tumour suppressors or oncogene proteins which can result in loss of control of cellular proliferation leading to the development of tumours [86]. This is evident in studies that have shown increased rates of protein synthesis in breast and colon tumours compared to normal tissues [136] and increased levels of eIF2-α are seen in certain cancer cells, including malignant melanomas, adenoma carcinomas of the colon, and the majority of aggressive non-Hodgkin's lymphomas [137]. However, what it is not clear is whether this is a cause or a consequence of transformation [138]. Examining various gastrointestinal neoplasm, Lobo *et al* [139] showed that the levels of phosphorylated eIF2-α in tumour cells compared to normal tissue are elevated and this can be due to activation of ISR within the tumour itself where cells are subjected to hypoxia and/or nutrient deprivation. Another suggestion is that tumour cells can tolerate higher levels of p-eIF2-α, due to elevated levels of eIF2B [138]. A number of mitogenic signals, proto-oncogenic and tumour supressors regulate the rate of rRNA synthesis and hence protein synthesis via upstream binding factor (UBF), a regulator of RNA polymerase I [140]. In turn UBF is upregulated by ERK, AKT, PI3K and mTOR, key cellular signaling proteins which are frequently activated in tumours [141, 142]. These examples provide links between signals involved in tumourigenesis and control of translation.

Targeting translational machinery with drugs has emerged as a very valuable approach for cancer treatment. One successful example is L-asparaginase, which is a standard treatment of leukemia [143]. Transformed hematopoietic cells cannot synthesize sufficient levels of asparagine for their own metabolism; consequently they rely on serum asparagine, which can be depleted by administration of L-asparaginase causing inhibition of protein synthesis and apoptosis in leukemic cells [144]. Other examples of translation inhibitor drugs are: Bouvardin

for treatment of leukemia [145], emetine and cyclohexamide as potentiators of doxorubicin apoptotic effects to treat HPV E6 oncogene-induced drug resistance [146], and rapamycin which is used to inhibit translation initiation [147].

Histone deacteylase inhibitor, SAHA, potentiates cisplatin-induced apoptosis in oral squamous cell carcinoma, however following treatment with salubrinal, the small molecule inhibitor of eIF2-α dephosphorylation, this additive effect was abrogated, suggesting that the ISR is an integral part of this chemosensitization event [148]. In contrast by increasing phosphorylated of eIF2-α, salubrinal can eradicate bortezomib-treated surviving quiescent MM cells, which show attenuated p-eIF2-α levels [149]. Likely this is also the reason behind the observed synergism effect of proteasome inhibitors (PIs) and salubrinal in various examined MM cells [150], stressing both cytotoxic and cytoporotective roles of ISR. These data suggest that induction of phosphorylation of eIF2-α, at least for certain types of chemotherapies and/or cell types, may have therapeutic significance.

1.2.c ATF4

Phosphorylation of eIF2-α can regulate both gene-specific and general translation in eukaryotic cells [151]. As a result of phosphorylation of eIF2-α and reduced levels of TC, the rate of protein synthesis reduces however, paradoxically ATF4 translation is increased [105]. ATF4, also known as CREB-2 [152] is a member of the CREB (cyclic AMP response element binding protein) transcription factor family of basic leucine zipper (bzip) transcription factors. The *ATF4* gene contains two uORFs [153]. When levels of the non-phosphorylated eIF2-α and consequently, TC, are high ribosomes scanning through *ATF4* mRNA translate the first ORF and are also capable of reinitiating at the second uORF, which blocks the translation of ATF4. Under

stress conditions the phosphorylated form of eIF2-α is high and the levels of TC are lower thus a major portion of the scanning ribosomes are not competent to reinitiate at the second ORF, therefore bypassing it and allowing initiation at the main *ATF4* start codon [124].

Expression levels of ATF4 are enhanced in response to several stress conditions such as oxidative stress, UPR, nutrient deprivation and hypoxia [154]. Additionally, ATF4 modulates the expression of genes involved in metastasis, angiogenesis, differentiation, and long-term memory [155]. A recent study shows that stabilization of basal levels of ATF4 protein-in part independent of PERK activity- has a critical role in MCF-7 resistance to bortezomib induced cell death [156]. A novel function of ATF4 has been identified in mediation of the induction of 4E-BP1, the suppressor of the mRNA 5' cap-binding protein eukaryotic initiation factor 4E (eIF4E) where it contributes to the loss of pancreatic β-cell mass due to activation of ER stress, suggesting a broader translational regulatory role for induction of ISR [157].

ATF4 has various binding partners for example, CHOP (discussed below), to which it binds to under conditions of amino acid deprivation leading to suppression of asparagine synthase (ASNS) [158, 159]. ATF4 targets also include: NRF2, the novel substrate of PERK and transcription factor that regulates expression of anti-oxidant, cytoprotective and anti-xenobiotic genes [160]; SKIP3/TRB3, a human kinase-like protein which is overexpressed in several tumours [161];proteins involved in amino acid transport and metabolism [162]; and ATF3 [163]. The diversity of ATF4 binding partners suggests a broad range of functions for this transcription factor [163]. Further, ATF4 is known as a transcriptional activator of several genes, including, *CHOP* [164] , *GADD45* [165], and *ASNS* [166] under various stress conditions. Expression of ATF4 is mainly correlated with translation regulation however, increases in *ATF4* mRNA levels

by glucose and amino acid deprivation [167] , anoxia [168] and by heregulin (HRG) β-1, a ligand for EGFRIIV and –IV in cancer cells, have been reported [169] .

Primary tumour tissues show elevated levels of ATF4, due to hypoxia/anoxia, compared to normal tissue where it plays a role in enhancing tumour growth in xenograft models [163]. Additionally, ATF4 enhances the expression of VEGF, a potent stimulator of angiogenesis [170] Chromatin immunoprecipitation (ChIP) analysis, which identifies promoter regions within the genome that a protein interacts with, showed that CHOP was associated with the CCAAT/enhancer-binding protein (C/EBP)-ATF composite site region of *VEGF*, suggesting that induction of ATF4-CHOP is involved in regulation of angiogenesis via affecting VEGF [170]. Interestingly microarray expression analysis of the HNSCC cell lines SCC9 or SCC25 following treatment with 10 μM lovastatin for 24 hrs showed that *ATF4* was among the 9 cDNAs affected in both cell lines [43].

1.2.d CHOP

CHOP, the first molecule implicated in ER stress induced cell death, is a transcription factor that in the absence of stress is expressed at very low levels but is strongly induced by ISR and is, more specifically, a downstream target of p-eIF2α [171]. Following stress induction CHOP migrates to the nucleus where it mediates the expression of its target genes [172]. Several examples of stress inducers that can enhance CHOP expression are nutrient deprivation, such as glucose or amino acid deprivation; agents that can disturb calcium homeostasis of the ER e.g. TG; DTT due to disruption of protein disulfide bond formation; and brefeldin A through disruption of intracellular trafficking. *CHOP* has at least two ER stress response elements

(ERSE) within its promoter region and two amino acid regulatory elements (AARE)[171]. During arsenite treatment ATF4 and ATF3 bind consecutively to the AARE promoter of *CHOP* as activator and suppressor respectively [164, 173]. Deletion studies have shown that CHOP protects cells against ER stress-induced apoptosis while its over-expression leads to increased sensitivity to ER stress induced apoptosis [174]. CHOP is also implicated in the antitumour function of cyclooxygenase-2 (COX-2) inhibitors (e.g. Celecoxib) [175, 176], curcumin [177] and fenretinide [178] , and its expression is also enhanced by taxol and cisplatin [179]. One of the target genes of CHOP is *GADD34* which, through a negative feedback loop, is involved in the de-phosphorylation of eIF2-α and controls the induction of ISR in affected cells. Furthermore, the phosphatase activity of the GADD34 complex in CHOP null MEFs is attenuated and consequently less proteins are synthesized in CHOP null MEFs compared to the wild type , which in turn challenges the ER of stressed cells with less load of proteins to avoid the lethal consequences of ER stress [180]. Consistently, the small molecule inhibitor, salubrinal, which inhibits de-phosphorylation of eIF2- α can protect cells against ER stress-induced cell death [181].

Studies of the induction of CHOP focus mainly on the PERK/p-eIF2α/ATF4/CHOP cascade. In this context, the higher levels of p-eIF2-α are associated with less protein synthesis and higher levels of chaperone reserve which in certain conditions can promote cell survival [86]. There is a suggestion that CHOP, by promoting apoptosis in damaged cells, might facilitate the regeneration of tissue and help to maintain the survival of the organism [86]. In certain cellular contexts, CHOP suppresses expression of anti-apoptotic BCL-2 leading to increases in free oxygen radicals followed by activation of mitochondrial dependent apoptotic pathways [79, 174] and converseley enhances the expression of proapoptotic death receptor 5(DR5) in colon

cancer cells following TG treatment [182]. Another target gene of CHOP is *ERO1α*, an ER oxidase enzyme, involved in maintenance of redox homeostasis within the ER [183]. Overall, the role of CHOP in cancer cells is not well understood, as its effects may be nullified by downstream mutations in apoptotic machinery at the levels of inhibitors of apoptosis (IAPs) [184] or Caspases 3 and 9 [79, 185].

Although the majority of reports suggest a pro-apoptotic role for CHOP, there are reports indicating cyto-protective activities. Expression of CHOP protects against cell death of oligodendrocytes in Pelizaeus-Mezbacher disease where, due to a mutation in proteolipids, protein misfolding occurs, activating ER stress [186]. Another study indicates a similar role for CHOP against radiation-induced apoptosis in thiol depleted lymphoma cells [187]. A possible explanation for the cytoprotective role of CHOP is its ability to promote protein synthesis, for example through upregulation of GADD34, and this could promote survival through modulation of ISR activation, rather than death [86]. Deletion studies have shown that the bzip domain of CHOP is essential for its ability to induce apoptosis [188]. Through this domain CHOP can form hetero or homo-dimers with other members of bzip transcription factor family [189]. It has been suggested that inhibition of cell death by CHOP may occur through binding with C/EBP-β and coupling of the activation of the prosurvival AKT/NF-κβ pathway with induction of BCL-2/xl [190]. This suggests that the function of CHOP to mediate apoptosis is cell-type specific and different effects are exerted through activation of various downstream genes [190].

1.2.e ATF3

ATF3 is also a member of the ATF/CREB family of transcription factors and is induced by various stress signals including ischemia, chemical exposure-including alcohol and

acetaminophen, exposure to cytokines, and UV or ionizing radiation [191]. It can form homodimers, where it acts as an transcriptional repressor, even for its own promoter [192]. However it can also form heterodimers with other CREB family members such as ATF4 [193] and CHOP to either activate or repress transcription [191]. ATF3 binding also stabilizes p53 in response to DNA damage [194]. In terms of transcriptional regulation, several target genes of ATF3 include *CHOP*[195], asparagine synthase (*ASNS*) [164], as well as several genes identified in a breast cancer cell line that are implicated in tumour metastasis; including fibronectin (*FN*)-1, *TWIST1*, caveolin-1, plasminogen activator inhibitor-1 and *SLUG* [196]. During ISR, depending on the stress condition, expression of ATF3 can be enhanced by ATF4 leading to transcriptional regulation of *CHOP*. Deprivation of amino acids, a condition that also activates GCN2, causes ATF3 to bind to the promoter of *ASNS* modulating its expression coordinately with ATF4 [166].

The wide range of stress conditions involved in induction of ATF3 suggest different signaling pathways are involved in regulating the expression of this transcription factor [197] [192]. In addition to activation by ATF4, the promoter of *ATF3* is also shown to be activated by ATF2 and c-Jun, two downstream targets of mitogen-activated protein kinase (MAPK) [198]. Under certain stress conditions ATF3 can be induced independent of ISR, through other signaling pathways, such as p38, thus mediating p38 induced cell death [199]. Inhibition of this pathway prevented induction of ATF3 by several stress inducers, such as anisomycin (a *Streptomyces griseolus* antibiotic known to inhibit protein synthesis), interlukin-1-β (IL1-β), TNF-α and H_2O_2 [197, 200].

ATF3 expression is not exclusively stress dependent. During normal cellular functions such as growth, proliferation and motility ATF3 can also be induced. For example ATF3 is

induced in MCF-7 breast cancer cells by adipokines, the secreted factors from adipocytes, which promote cell survival and motility [201]. ATF3 is also induced during S phase and can affect both cell cycle progression and survival [202, 203]. ATF3 null MEFs transition from G1 to S phase more efficiently than wild-type and overexpression of ATF3 slows down this transition, suggesting a cell cycle arrest role for ATF3 [194, 204]. Consistent with this notion, upon Ras transformation, ATF3 null MEFs showed faster growth in soft agar, with more colonies, and formed larger tumours in xenograft models, compared to wild-type MEFs [205]. Other studies also observed induction of ATF3 as a result of anoxia/hypoxia in a p53 and HIF1α-independent manner [97]. Interestingly, following UV irradiation, ATF3 expression is enhanced independent of GCN2 and ATF4. GCN2, under these conditions, is shown to play an anti-apoptotic role [106]. These data suggest that induction of ATF3 by UV is independent of ISR and occurs through another signaling pathway. In light of these studies it has been suggested that ATF3 is an adaptive response gene rather than simply a stress-inducible gene, as it exerts its effects in a wide variety of cellular processes [197].

Depending on the stress inducer and/or cell type there are reports that suggest that ATF3 has either an oncogenic or a tumour suppressor role. Increased ATF3 expression, due to lower expression of its modulator, the tumour suppressor gene, *DRG*-1, is positively correlated with metastasis in prostate cancer cell lines [206]. Conversely, one of the target genes that is repressed by ATF3 is E-selectin [207] , a cell surface adhesion molecule implicated in tumour metastasis [208]. ATF3 also represses matrix metalloproteinase (*MMP*)-2, another factor implicated in the angiogenesis and invasion of malignant tumours [209, 210].

The dual functionality of ATF3 in prosurvival or death mechanisms during stress activation may be related to regulation through alternative promoters of the *ATF3* gene [211] . In

prostate and Hodgkin Reed-Sternberg cancer cells one of these promoters was constitutively active resulting in elevated levels of ATF3 [211, 212]. Additionally, in one study, more than two copies of *ATF3* were detected in more than 80% of breast tumours [151]. Regardless, these data underline the requirement for further studies of the role of ATF3 in a stimulus and cancer cell dependent manner for efficacy of treatment.

1.2.f ISR as a Target/Tool for Cancer Therapy

Activation of the UPR in all cases may not be to the cell or organisms benefit and can also be harmful, by either promoting cell death or by protecting an unhealthy cell from apoptosis. The protective role of ISR during neoplastic transformation and hypoxia in tumours are examples where this response is harmful [96, 213]. ER stress and CHOP induction have also been implicated in plaque necrosis in advanced atheroslerotic lesions [214] .

Hypoxia is a common condition of many solid tumours, due to the formation of areas of poor vascularisation within the tumour. There is molecular evidence of ISR activation (including expression of ATF4 and CHOP in tumour biopsy samples) in several human tumours, including melanoma, breast, glioblastoma and cervical cancers [96]. These studies suggest a model by which tumour cells activate ISR to enhance viability under hypoxic conditions by reducing translational activity, thereby reducing metabolic requirement of the cell, and secondly promoting tumour growth by increasing angiogenesis in response to low oxygen levels [215]. Several components of the UPR, including XBP-1 [216] and the ER chaperone BiP/GRP78 [217] have also been shown to be upregulated in many cancers, most likely as a means to adapt to oxygen/nutritional deprivations in the microenvironments of tumours. GCN2 also has recently being explored as a novel G1/S transition checkpoint factor, following UV irradiation and this activity is tightly correlated with phosphorylation of eIF2-α [218]. While induction of ISR due

to hypoxia in tumour cells may provide a means of survival under certain stress conditions, it is also possible that further enhancing of this response by agents which also induce ISR may push the cells response from survival mode to apoptosis.

It has also been proposed that phosphorylation of eIF2-α may directly, or indirectly, regulate select proteins that are crucial for cell growth and proliferation [219]. Thus, chemotherapeutics that are capable of inducing ISR may interfere with these cellular processes, providing mechanisms to control growth and proliferation of tumours. Anti-cancer drugs that have been demonstrated to induce ISR include cisplatin, brefeldin A, gleevec and more recently versipelostatin [220-223]. ISR activation is also essential for bortezomib induced apoptosis in HNSCC [224] and interestingly, cooperative effects of this drug for inducing ER stress in conjunction with celecoxib enhance apoptosis in glioblastomas [225]. Combinations of ISR inducing agents could therefore also be used to effectively trigger cell death by eliciting higher cytotoxic outputs from this pathway.

1.2.g UPR and Mevalonate pathway

Cellular levels of cholesterol are regulated through the sterol regulatory binding protein (SREBP) family of transcription factors [226]. When the cell has sufficient levels of cholesterol SREBPs are embedded within the lumen of the ER, and upon depletion of cholesterol, they migrate to the Golgi-apparatus where they are cleaved by S1P and/or S2P, two type I proteases that also responsible for cleaving ATF6 [227]. Activated SREBPs are exported to the nucleus where they enhance the transcription of *HMG-CoA* reductase driving the mevalonate pathway and cholesterol biosynthesis [228]. SREBPs are also implicated in the regulation of fatty acid synthesis [228] . TG induced ER stress can activate SREBPs and this is correlated with the

degradation of ER resident protein, INSIG-1, a major modulator of HMG-CoA reductase in mammalian system [229, 230]. However, the molecular mechanism which transmits ER stress signals to SREBP is unknown. Since conventional UPR is activated by unfolded proteins, it is conceivable that changes in cholesterol homeostasis either directly or indirectly- through secondary mechanisms, exert an effect on protein folding in the ER.

There is emerging data linking disruption of cholesterol homeostasis to induction of ISR. In advanced atherosclerotic lesions, a study showed that accumulation of cholesterol in the ER of macrophages induced the UPR and activated CHOP mediated apoptosis, causing exacerbation of the lesion and plaque destabilization [231]. In addition, excessive cholesterol is also shown to deplete ER Ca^{+2} stores, thereby activating UPR [232]. Free fatty acids (FFA) are toxic to pancreatic islet cells, inducing ER stress mediated cell death which contributes to the development of type 2 diabetes [233]. A follow-up to this study showed that saturated FFA can cause UPR by sustained depletion of ER calcium stores and unexpectedly, ATF3 plays either a neutral (in mouse islets) or anti-apoptotic role (rat β-cells), while CHOP had only a pro-apoptotic role [234]. A novel function for GCN2 the regulation of lipid metabolism in leucine-deprived mice, independent of amino-acid deprivation activation of GCN2 was also reported [235]. The physiological role of this regulation, however, is unclear. PERK is also shown to positively regulate the amount of lipids in mammary epithelial cells and the levels of FFA content in the milk of conditional knockout mice was significantly reduced [236]. Moreover, *in vitro* analysis in PERK null MEFs vs. wild type shows that PERK regulates lipogenic maturation, through regulation of SREBP1 activation [47].

1.2.h. ER stress and calcium regulation

Perturbations to homeostasis of the ER, which contains the highest concentrations of intracellular calcium, can lead to release of calcium from the ER followed by apoptosis in a UPR dependent or independent manner [79]. Calpains are calcium-dependent cysteine endopeptidases that act as modulators of protein substrates within the cell. Disturbances of intracellular calcium levels for example, due to ischemic injury or cytotoxicity associated with β-amyloid, which induces UPR, activate calpains [237]. Nakagawa et al. reported that m-calpain may be involved in activation of ER-resident Caspase 12 and inactivation of anti-apoptotic BCL-xl protein, leading to induction of cell death in murine cells [237, 238]. The role of Caspase 12 in ER stress induced cell death is controversial. *Caspase 12 −/−* mice have shown contradictory responses to ER-stress induced cell death [151]. Xue et al. have shown that Caspase 12 is inactive in humans[239]. There have been suggestions that Capsase 4 or 11 are paralogues of murine Caspase 12 in humans, however, this has yet to be fully demonstrated and requires further studies[151]. Overexpression of IRE1-α is also associated with Caspase 12 induced apoptosis [240]. Activation of Caspase 12 is abolished in *Bax/Bak* double knockouts, but through reconstitution of ER targeted BAK, Caspase 12 processing was restored, due to ER Ca^{2+} depletion [241]. In addition, activation of Caspase 9 during ER stress, independent of mitochondrial cytochrome C and APAF1, suggests that Caspase 12 might directly process Caspase 9 and trigger apoptosis. Intriguingly, other studies indicate the involvement of mitochondria in ER stress-induced apoptosis, suggesting a redundant or independent role for Caspase 12 during ER stress [242].

1.3 Rationale and Hypothesis;

Lovastatin induces apoptosis in a specific subset of tumour cells. However Phase I and II clinical trials using high dose statin regimens showed limited clinical activity. The molecular mechanism of induction of apoptosis in sensitive cell lines generally by statins and more specifically lovastatin, is not very well understood. Therefore, examining the molecular mechanism by which lovastatin and similar agents selectively promote cellular death in cancer cells will help to facilitate development of more effective treatments whereby lovastatin can be utilized in combination with agents which potentiate its anticancer activities.

Inhibition of the mevalonate pathway causes disruptions of intracellular trafficking by abrogating the function of Rab proteins. This can cause accumulation of non-processed proteins in the ER and/or limit the levels of accessible amino acids for translation machinery. Furthermore, an increase of intracellular calcium levels following statin treatment in several cell lines is a condition that is associated with induction of ER dysfunction. Altogether these data suggest that lovastatin may causes induction of ISR in sensitive cell lines.

Hypothesis

Lovastatin, through its ability to affect various cell signaling pathways, will induce cellular stress responses such as the ISR and combination of lovastatin with other drugs which have similar targets will provide a novel therapeutic strategy. This hypothesis will be tested by dissecting the mechanism of action of lovastatin induced cytotoxicity and particularly;

1. Examining the role of the ISR in lovastatin-induced apoptosis by evaluating potential activation of the components of the ISR/UPR stress pathways following lovastatin treatment in sensitive cell lines.

2. Evaluating the role of downstream target ATF3 in this pathway using genetic knock-outs and mRNA depletion, through targeted shRNAs of this gene, in cancer cell lines.
3. Studying the role of calcium regulated genes within the context of lovastatin induced cell death.
4. Developing new therapeutic approaches by finding selective targets of lovastatin.

Chapter 2

ACTIVATION OF THE INTEGRATED STRESS RESPONSE REGULATES LOVASTATIN INDUCED APOPTOSIS

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Contribution of authors

This manuscript was written by Nima Niknejad and edited by Dr. Jim Dimitroulakos. All experiments were performed by Nima Niknejad with the exception of Figures 5-7 which were done with the technical assistance of Melissa Morley (former lab technician J.Dimitroulakos lab).

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Abstract

Lovastatin, a potent inhibitor of mevalonate synthesis, can readily induce apoptosis in a subset of human tumour types including head and neck squamous cell carcinomas (HNSCC). We recently identified activating transcription factor (*ATF*) 4 as a lovastatin induced gene in HNSCC cells. ATF4 plays a significant role in regulating cellular responses to a wide variety of stress inducers known as the integrated stress response (ISR). These cell stresses lead to the phosphorylation of eukaryotic initiation factor (eIF) 2α shutting down global protein translation. However, the translation of ATF4 is enhanced. In this study, lovastatin treatment induced eIF2α phosphorylation and inhibited global protein translation. ATF4 expression was induced followed by increased ATF3 and CHOP expression, targets of ATF4 activity, in SCC25 HNSCC cells. In CHOP-/- murine embryonic fibroblasts (MEFs), lovastatin-induced apoptosis was attenuated indicating a role for CHOP in this response. Furthermore, the eIF2α kinase GCN2 mediates lovastatin's induction of ATF4 and lovastatin-induced apoptosis was also attenuated in GCN2-/- MEFs. The pro-drug version of lovastatin has potential proteasome inhibitory activity and recently a variety of well established proteasome inhibitors were shown to activate the ISR. In this study, neither the pro-drug nor the active forms of lovastatin had any significant effect on proteasome activity. Therefore, lovastatin, by targeting mevalonate synthesis, is a potent inducer of the ISR through a novel and as yet unrecognized mechanism.

Introduction

The rate-limiting step of the mevalonate pathway is the conversion of HMG-CoA to mevalonate, which is catalyzed by HMG-CoA reductase [1]. The mevalonate pathway produces various end products that are critical for many different cellular functions. These products are

essential in maintaining cellular membrane structure and integrity, N-linked protein glycosylation, mitochondrial respiration, tRNA production and the prenylation of a wide variety of cellular proteins [1]. These prenylated proteins include many GTP-binding proteins such as members of the Ras, Rab, and Rho families. These proteins regulate cell proliferation, intracellular trafficking and cell motility [2,3] and this post-translational modification functions as a membrane anchor critical for their activity. Blockade of the rate-limiting step of the mevalonate pathway by HMG-CoA reductase inhibitors results in decreased levels of mevalonate and its downstream products [4] and, thus, may have significant influences on many critical cellular functions.

The family of statin drugs are potent inhibitors of HMG-CoA reductase widely used as hypercholesterolemia treatments [4]. Malignant cells appear highly dependent on the sustained availability of the end products of the mevalonate pathway [5]. Recent analyses have demonstrated that statin treatment can directly block tumour cell growth, invasion and metastases both *in vitro* and *in vivo* [6,7]. In initial clinical analyses of statins as anti-cancer therapeutics, no significant anti-tumour responses were observed [8,9]. New optimism regarding the use of statins, however, has emerged from recent studies. We and others have demonstrated that a number of human tumour types including squamous cell carcinoma of the head and neck (HNSCC), are particularly susceptible to lovastatin-induced apoptosis [10-12]. The identification of specific tumour types that are sensitive to lovastatin-induced apoptosis, spurred further clinical evaluation by our group and a Phase I trial in recurrent HNSCC and cervical carcinoma patients was undertaken. Although no tumour regressions were observed, 23% of patients exhibited stable disease suggesting further clinical evaluation is warranted [13]. Understanding

the mechanism of cellular apoptosis induced by lovastatin may uncover novel therapeutic approaches.

A variety of cell stressors result in the phosphorylation of eukaryotic initiation factor (eIF) 2α, which causes global translation attenuation [14]. The α subunit of eIF2 is the target of a family of serine or threonine kinases that are activated by different forms of environmental stress and include PKR, PERK, GCN2 and HRI [15]. For example, PKR senses viral infection [16], PERK detects endoplasmic reticulum (ER) stress and oxidative stress [15], GCN2 senses amino acid starvation [17] and HRI monitors changes in the availability of heme during erythrocyte differentiation [18]. Each of these stress-activated kinases phosphorylates eIF2α on serine 51, a modification that prevents the assembly of the pre-initiation complex, and halts global protein translation to aid in the cellular response to these stresses [14].

Under these conditions, however, the translation of activation of transcription factor (ATF) 4 is enhanced due to delayed translational re-initiation at upstream reading frames located at the 5'-end of the *ATF4* mRNA [19]. We recently identified *ATF4* as a lovastatin induced gene in HNSCC cells [20]. ATF4 is induced in response to a wide variety of cellular stresses playing a critical role in regulating this integrated stress response (ISR) [19]. Depending on the strength of the stress stimulus, these mediators can either alleviate the stress or alternatively induce apoptosis. Enhanced expression of the transcription factors *ATF3* and *CHOP*, ATF4 target genes, can mediate this apoptotic response [21]. The closed ring prodrug form of lovastatin is readily converted, both chemically and enzymatically, to its open ring active form that inhibits HMG-CoA reductase activity [4]. A few studies have shown that the prodrug form of lovastatin can inhibit proteasome function [22]. Proteasome inhibitors are potent inducers of the ISR likely due to the accumulation of misfolded proteins [23]. Upon chemical conversion of the prodrug to the

active form of lovastatin, a residual concentration (up to 20%) of the prodrug can remain [22]. Therefore, there is the potential for lovastatin to trigger the ISR through proteasome inhibition.

In this report we evaluated the potential of lovastatin to induce the ISR in HNSCC cells. We further characterized the role of the eIF2α kinases PERK and GCN2 as potential mediators of lovastatin-induced apoptosis. We also evaluated the role of proteasome inhibition in regulating this response. Delineating the mechanism of lovastatin-induced apoptosis may lead to more refined therapeutic approaches using this class of agents.

RESULTS

Lovastatin Induces eIF2α Phosphorylation

eIF2α plays a significant role in regulating the ISR [14]. A variety of kinases activated by cellular stresses phosphorylate eIF2α shutting down global protein translation to allow for recovery from the insult [15]. SCC25 cells were treated with 10 and 50uM lovastatin for 3, 12 and 24hrs as well as with 1μM thapsigargin (Tg) for 24hrs as a control. Tg is a potent inhibitor of the ER resident calcium ATPase that results in calcium accumulation in the cytoplasm through the depletion of ER stores leading to ER stress [27]. Phosphorylation of eIF2α at serine 51 was detected by Western blot analysis and compared to total eIF2α expression. The phosphorylation of eIF2α was enhanced with both lovastatin concentrations with Tg showing a more pronounced response (Figure 1A). In similarly treated SCC25 cells, we were able to demonstrate global protein translation attenuation as lovastatin treatments dramatically reduced ^{35}S-methionine labeled incorporation into newly synthesized proteins (Figure 1B). Taken together, these results

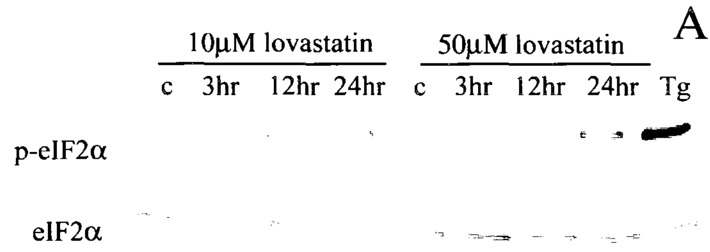
demonstrate that lovastatin induced the phosphorylation of eIF2α at functional levels that resulted in the shutdown of global protein translation, hallmarks of the cellular ISR [14].

In a previous study, we had demonstrated that the transcription factor *ATF4* was up regulated in lovastatin treated HNSCC cell lines [20]. Expression levels of ATF4 are enhanced as a result of the phosphorylation of eIF2α [19]. With the same concentration and time-course used above in SCC25 cells, we demonstrated a significant induction of ATF4 protein in these lovastatin treated cells by Western blot analysis (Figure 1C). This was accompanied by a relatively modest increase (<3fold) of mRNA levels for *ATF4* as determined by real time RT-PCR under similar experimental conditions and in the range of Tg treatment (1μM, 24hrs) (Figure 1D). The increase in ATF4 protein levels in SCC25 lovastatin treated cells was likely at the level of protein translation as mRNA levels showed only modest changes.

Lovastatin Induces ATF3 and CHOP Expression

ATF4 is a member of the ATF/CREB family of transcription factors [28]. Consensus ATF4 binding sites are present on the promoters of a number of genes that play a role in regulating cellular stress responses including *ATF3* and *CHOP* [28]. Expression of these genes has previously been shown to be regulated in part by ATF4. We evaluated the effects of lovastatin treatment on the expression of *ATF3* and *CHOP* in SCC25 cells. Western blot analysis demonstrated enhanced ATF3 protein expression at 24hrs 10μM lovastatin treatment (Figure 2A). Using real time RT-PCR, 10μM lovastatin induced a 10 fold increase in *ATF3* mRNA levels by 24hrs treatment (Figure 2A). The 50μM lovastatin treatments induced both mRNA and protein levels at earlier time points and at higher levels. These results demonstrate that ATF3 expression is regulated by lovastatin treatment in SCC25 cells.

Figure 1. Lovastatin induces phosphorylation of eIF2-α and ATF4 and inhibits cellular protein synthesis. A, Lovastatin induces phosphorylation of eIF2-α and ATF4 and inhibits cellular protein synthesis .Western blot analysis of phosphoserine 51 eIf2α and total eIF2α in SCC25 cells after lovastatin treatment. Cell lysates from 10 and 50μM lovastatin treatments for 3, 12, and 24hr as well as solvent control (c) and 1μM thapsigargin (Tg) for 24hr were analyzed. Levels of total eIF2α were assayed as the loading control. B, Translational attenuation following lovastatin treatment in SCC25 cells. SCC25 cells were treated with 10 and 50μM lovastatin for 3, 12, and 24hr, as well as solvent control (c) and 1μM thapsigargin (Tg) for 24hrs. Newly translated proteins were labeled with [^{35}S]Methionine incorporation for 15min and cell lysates were prepared and analyzed by SDS-PAGE and autoradiography. C, Western blot analysis of ATF4 protein levels in SCC25 cells after lovastatin treatment. Cell lysates from 10 and 50μM lovastatin treatments for 3, 12, and 24hr as well as solvent control (c) and 1μM thapsigargin for 24hr were analyzed. Expression levels of actin were assayed as the loading control. D, Levels of *ATF4* mRNA were analyzed by real time quantitative PCR using similar conditions as the Western blot analyses described above. Fold changes were calculated following normalization to *GAPDH* levels (ΔΔCt) and expressed as means (+/- SEM) (n=4).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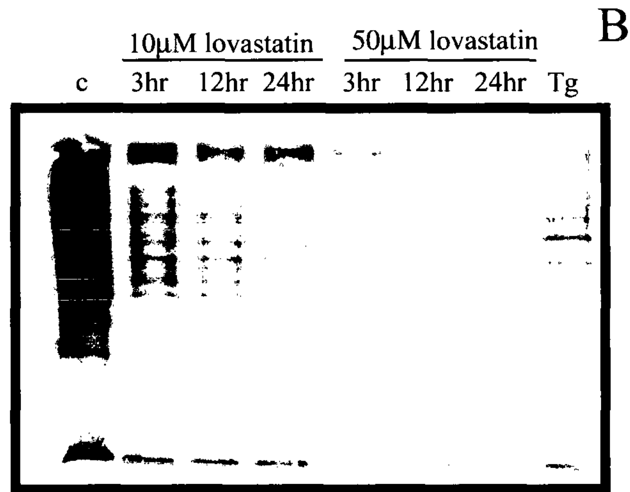
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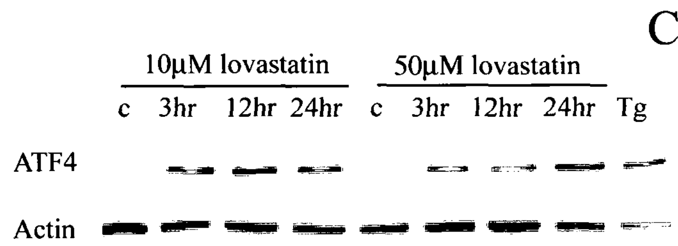
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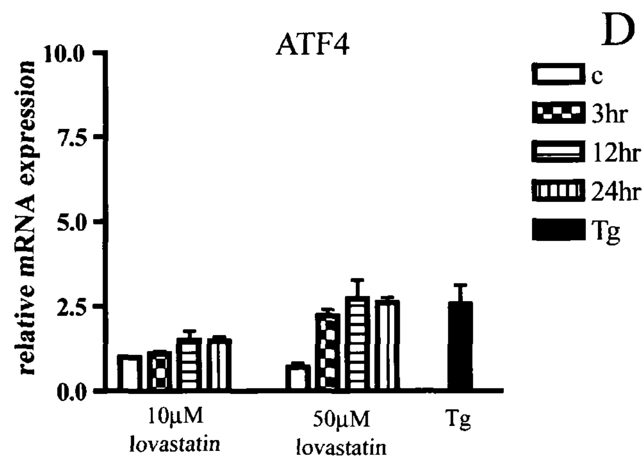
D
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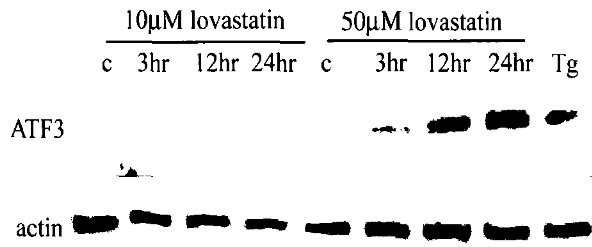
24hr
Tg

CHOP is another member of the ATF/CREB family of transcription factors that plays a role in the induction of apoptosis when cellular stress cannot be controlled adequately [29]. CHOP can modulate the expression of a number of apoptosis regulating genes [30]. CHOP protein and mRNA levels were significantly elevated within 12hrs of 10μM lovastatin treatment of SCC25 cells (Figure 2B). At the higher 50μM dose, *CHOP* mRNA and protein levels were up regulated earlier (3hrs) and to higher levels. To determine if this increase in CHOP expression results in the transcriptional regulation of CHOP targets, we evaluated the expression of GADD34, which is a potent suppressor of ISR [243] . Consistent with other cellular stressors [28], GADD34 expression was up regulated by even 10μM lovastatin treatment (Chapter 3). These results demonstrate that CHOP expression is induced by lovastatin in SCC25 cells resulting in the activation of its transcriptional targets.

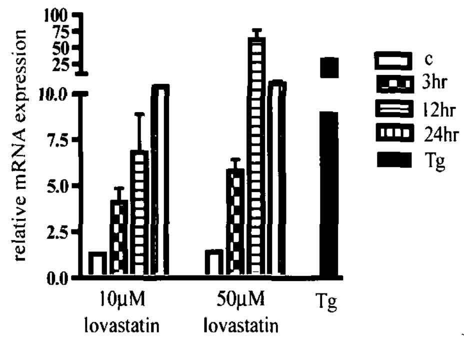
CHOP Regulates Lovastatin Induced Apoptosis

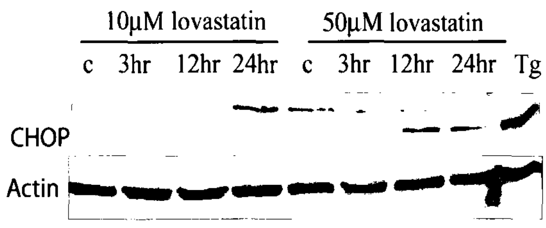
CHOP is implicated in apoptosis induced by the ISR [14, 31]. MEFs derived from *CHOP –/ –* animals display an attenuated apoptotic response when challenged with agents that induce the ISR [32]. Since MEFs are responsive to lovastatin-induced apoptosis, we used wild-type and *CHOP-/-* MEFs as a model system to evaluate the role of this transcription factor in lovastatin-induced apoptosis. MTT viability assays were performed 48hrs after 0.1-25μM lovastatin treatments. Analysis of these data showed attenuation in CHOP-/- MEFs of lovastatin-induced cytotoxicity (Figure 3A). Flow cytometric analysis using PI staining to determine the effect on the subG1 apoptotic population induced by lovastatin was employed. After 24hr and 48hr lovastatin treatments at 10μM, there was a significant reduction in the sub-G1 population in

Figure 2. Lovastatin induces ATF3 and CHOP in SCC25 cells . A,Western blot analysis of ATF3 protein levels in SCC25 cells following lovastatin treatment. Cell lysates from 10 and 50μM lovastatin treatments for 3, 12, and 24hr as well as solvent control (c) and 1μM thapsigargin for 24hr were analyzed. Expression levels of actin were assayed as the loading control. Levels of *ATF3* mRNA were analyzed by real time quantitative RT-PCR using similar conditions as the Western blot analyses described above. Fold changes were calculated following normalization to *GAPDH* levels ($\Delta\Delta$Ct) and expressed as means (+/- SEM) (n=4). B, Western blot and real time quantitative RT-PCR analyses of CHOP protein and mRNA levels in SCC25 cells following lovastatin treatment and analysis as above.

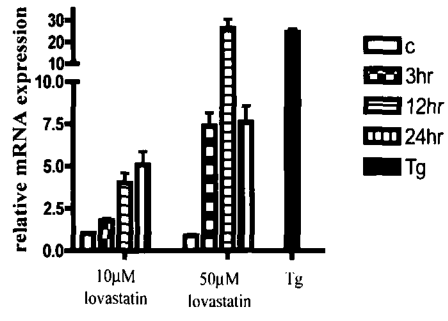


A





B

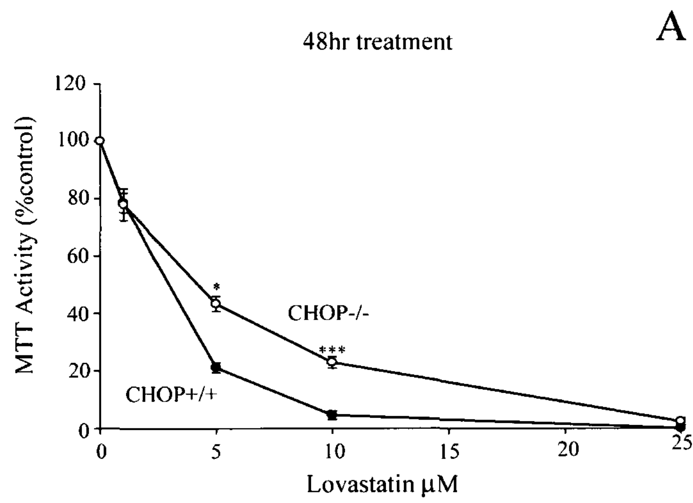


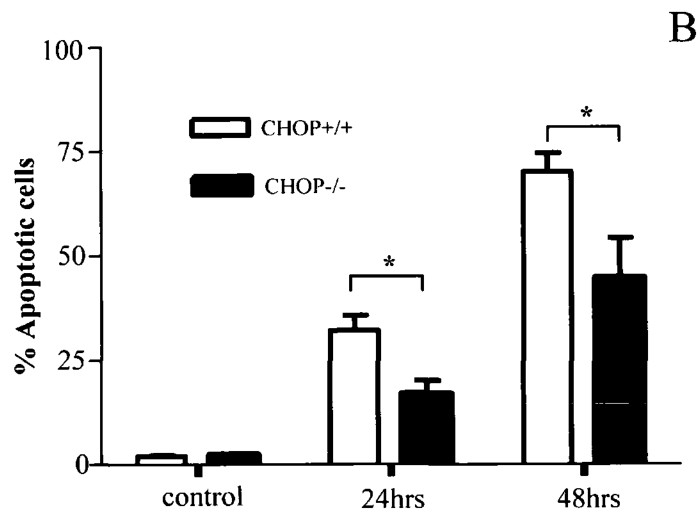
CHOP-/- MEFs compared to wild-type MEFs (Figure 3B). This differential response of CHOP+/+ and CHOP-/- MEFs to lovastatin treatment was also evident by observing changes in cell morphology. More significant cell rounding, typical of cells undergoing apoptosis, was evident in CHOP+/+ cells than in CHOP-/- cells treated with 10μM lovastatin for 12hrs (data not shown). This attenuation in cytotoxicity was also observed in CHOP-/- MEFS treated with 1μM Tg as has previously been reported [183] but not with staurosporin treatment (data not shown) whose cytotoxicity does not involve the induction of the ISR pathway. These results demonstrate a role for CHOP in the regulation of lovastatin-induced apoptosis.

GCN2 Mediates Lovastatin's Induction of ATF4

Phosphorylation of eIF2α at serine 51 results in global protein translation attenuation to allow for cell recovery from a number of cellular stressors [14]. The two relevant kinases that may regulate the ability of lovastatin to induce the ISR are PERK and GCN2 that are activated by ER stress and amino acid deprivation, respectively [15, 17]. Mevalonate metabolites play key roles in ER trafficking and tRNA synthesis [1, 33] and targeting this pathway with lovastatin may activate PERK and/or GCN2. In this study, we evaluated the ability of lovastatin treatment to induce ATF4 expression in MEFs lacking either PERK or GCN2 kinases. Treatments of wild type +/+, PERK-/- or GCN2-/- MEFs with 10μM lovastatin for 3, 12 and 24hrs were evaluated by Western Blot analysis for changes in ATF4 expression. Tg treatment (1μM for 24hrs) was also evaluated (Figure 4A). ATF4 expression was readily induced by 24hrs in lovastatin treated +/+ and PERK-/- MEFs. Tg induced ATF4 expression in +/+ but this response was attenuated in PERK-/- MEFs. GCN2 MEFs failed to induce ATF4 expression following 10μM lovastatin treatments while Tg readily induced ATF4 expression in these cells. To evaluate the roles of these eIF2α kinases in lovastatin induced cytotoxicity, MTT cell viability assays were performed

Figure 3. CHOP-/- cells are protected from lovastatin induced apoptosis. A, MTT viability assay comparing the response of CHOP-/- and CHOP+/+ MEFs to lovastatin treatment. CHOP-/- and CHOP+/+ MEFs were treated with 0-25μM lovastatin for 48hrs and cell viability was assessed with the activity of untreated cells taken to be 100%. Statistically significant differences were determined using one-tailed t-test (* $P<0.05$, ***$P<0.001$). B, Flow cytometric analysis of subG1 apoptotic fraction as determined by propidium iodide staining of cellular DNA content. CHOP-/- and CHOP+/+ MEFs were treated with 10μM lovastatin for 24 and 48hrs, fixed, stained with propidium iodide and analyzed by FACS. Percentages of sub-G1 cells are indicated. Statistically significant differences were determined using one-tailed t-test (* $P<0.05$).


48hr treatment
A
MTT Activity (%control)
120
100
80
60
40
20
0
*
***
CHOP-/-
CHOP+/+
0
5
10
15
20
25
Lovastatin μM

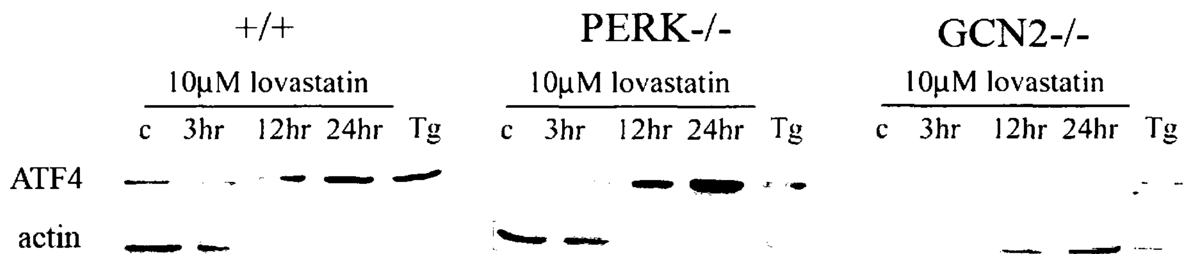

B
% Apoptotic cells
100
75
50
25
0
CHOP+/+
CHOP-/-
*
*
control
24hrs
48hrs

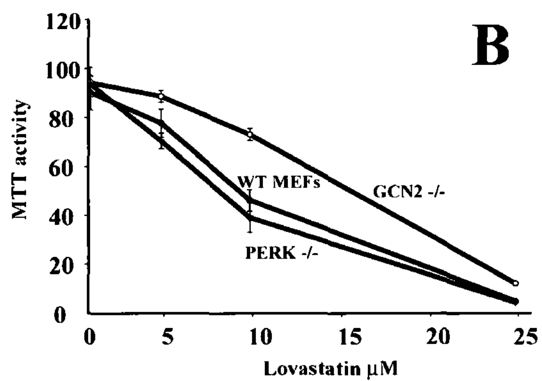
48hrs following 0.1-25μM lovastatin treatments of +/+, PERK-/- and GCN2-/- MEFs. Lovastatin treated +/+ and PERK-/- MEFs showed similar levels of cytotoxicity while the GCN2-/- MEFs displayed significant attenuation of the cytotoxic effects of lovastatin (Figure 4B). Taken together, these data demonstrate a role for GCN2 in regulating lovastatin-induced ISR and cytotoxicity in MEFs.

Lovastatin Fails to Inhibit Proteasome Function

Upon chemical activation of lovastatin to its open ring form that is a potent inhibitor of HMG-CoA reductase, up to 20% remains in the prodrug closed ring form that does not inhibit HMG-CoA reductase function [22]. Although controversial, various reports have shown that the prodrug form of lovastatin can inhibit proteasome function [22]. More recently, well established proteasome inhibitors like MG132, have been shown to activate the ISR likely through the accumulation of misfolded proteins [23]. In this study, we evaluated the potential of lovastatin to inhibit proteasome activity as a mechanism of its induction of the ISR. In this study, we compared the ISR induction of 25μM lovastatin to 1μM MG132 at 3, 12 and 24hr time-points in the HNSCC cell line SCC25. Both lovastatin and MG132 treatments induced ATF4, ATF3 and CHOP expression to similar levels in comparison to 1μM Tg treatments for 24hrs in SCC25 cells (Figure 5A). The heat shock protein (HSP) 70 is a major cytoplasmic chaperone protein whose expression is induced in response to accumulated misfolded proteins in the cytoplasm [34]. BiP is an abundant ER resident chaperone protein that facilitates proper protein folding and maturation within the ER and is induced under conditions of ER stress [35]. Proteasome inhibitors have been shown to induce the expression of HSP70 and BiP, with ER stress and BiP induction potentially occurring as a secondary event [23]. In this study, 1μM MG132 treatments induced HSP70 expression by 12hrs while BiP was only weakly induced by 24hrs (Figure 5A).

Figure 4. ATF4 induction by lovastatin is abrogated in GCN2-/- cells but not PERK-/- cells. A, Western blot analysis of ATF4 protein levels following lovastatin treatment of wild-type +/+, PERK-/- and GCN2-/- MEFS. Cell lysates from 10mM treatments for 3, 12 and 24hrs as well as solvent control (c) and 1mM Tg treatment for 24hrs were analyzed. Expression levels of actin were assayed as the loading control. B, MTT viability assays comparing the responses of wild-type +/+, PERK-/- and GCN2-/- MEFS treated with 0-25μM lovastatin for 48hrs and cell viability was assayed with the activity of untreated cells taken to be 100%.


A
+/+
PERK-/-
GCN2-/-
10μM lovastatin
10μM lovastatin
10μM lovastatin
c 3hr 12hr 24hr Tg
c 3hr 12hr 24hr Tg
c 3hr 12hr 24hr Tg
ATF4
actin


B
120
100
80
60
40
20
0
MTT activity
0
5
10
15
20
25
Lovastatin μM
WT MEFs
GCN2 -/-
PERK -/-

Autophosphorylation of IRE1, localized to the ER membrane, occurs in response to ER stress that results in activation of its endonuclease activity cleaving a 26 nucleotide segment of the *XBP1* mRNA [36]. This results in the translation of the full length activated version of this transcription factor that can enhance BiP expression [36]. Using RT-PCR analysis that delineated the unspliced from the activated spliced version of *XBP1* mRNA, MG132 was a weak activator of IRE1 while Tg induced significant *XBP1* mRNA splicing (Figure 5B). These results are consistent with previously published reports [23]. Lovastatin treatment on the other hand, failed to induce either HSP70 or BiP expression or IRE1 activation indicating that, unlike MG132, accumulation of misfolded proteins is not a mechanism by which lovastatin activates the ISR (Figure 5A and B).

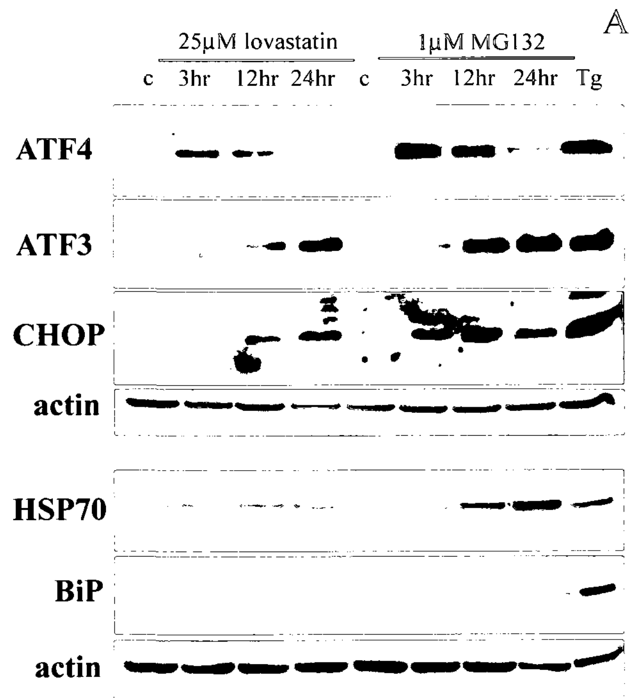
We next evaluated the ability of mevalonate co-administration to modulate the cytotoxic effects and the induction of the ISR by 25μM lovastatin, 25μM of its prodrug version and 1μM MG132. The prodrug version was dissolved in ethanol without chemical activation. Mevalonate co-administration reversed the cytotoxic effects of lovastatin (0-50μM for 48hrs) as determined by the MTT viability assay and also inhibited the activation of the ISR as induction of ATF3 and CHOP expression was abrogated (Figure 6A). Similar results were also demonstrated for the prodrug form of lovastatin (Figure 6B). In contrast, mevalonate co-administration had no effect on MG132 cytotoxic effects or its ability to induce a potent ISR in SCC25 cells (Figure 6C).

Proteasomes are multi-subunit proteases whose catalytic core shows peptidylglutamyl peptide hydrolyzing (PGPH), trypsin-like (TL) and chymotrypsin-like (ChTL) activities [37]. Employing three fluorogenic substrates that are specific for PGPH, ChTL and TL protealytic activity of the 26S proteasome, we evaluated the effect of lovastatin and its prodrug version on proteasome function. Proteasome cleavage of these fluorogenic substrates releases the AMC

fluorochrome that is subsequently detected and quantified using a fluorometer [37]. MG132 and PSI are potent inhibitors of ChTL activity but can also inhibit PGPH and TL activities [244]. After 24hrs of 10, 25 and 50μM treatments, neither lovastatin nor its prodrug form had any significant effect on proteasome activity (Figure 7A). Similar results were also observed following 3 and 12hr treatments (data not shown). Contrary to the effects of lovastatin and its prodrug version, 1μM MG132 and 5μM PSI showed significant inhibitory effects on ChTL, TL and PGPH proteasomal activities in SCC25 cells.

The proteasome recognizes and degrades proteins that have been modified through the covalent addition of ubiquitin chains [39]. Inhibition of proteasome function leads to the accumulation of polyubiquitinated proteins due to lack of degradation [39]. MG132 (1μM, 24hrs) and PSI (5μM, 24hrs) treatments of SCC25 cells lead to the accumulation of polyubiquitinated proteins demonstrated by Western Blot analysis. Both lovastatin and its prodrug version failed to induce the accumulation of ubiquitinated proteins (Figure 7B). Furthermore, exogenously expressing an unstable GFP construct that is readily degraded by the proteasome [25], similar treatments of MG132 and PSI treatments in 293T and HeLa cells inhibited GFP degradation, while lovastatin and its prodrug form had no effect on GFPu stabilization (Figure 7C). Taken together, these results clearly demonstrate that lovastatin does not affect proteasome function in these cells.

Figure 5. Analysis of downstream ISR effectors in lovastatin and MG132 treated SCC25 cells. A, Western blot analyses of the integrated stress response regulated proteins ATF4, ATF3 and CHOP as well as the chaperone proteins HSP70 and BiP following lovastatin and MG132 treatments of SCC25 cells. Cell lysates from 25μM lovastatin and 1μM MG132 treatments for 3, 12 and 24hrs as well as solvent control (c) and 1μM Tg treatment for 24hrs were analyzed. Expression levels of actin were assayed as the loading control. B, Total RNA was isolated from SCC25 treated cells as described above and unspliced and spliced versions of *XBP1* transcripts were assayed by RT-PCR analysis.


A
25μM lovastatin
1μM MG132
c 3hr 12hr 24hr c 3hr 12hr 24hr Tg
ATF4
ATF3
CHOP
actin
HSP70
BiP
actin

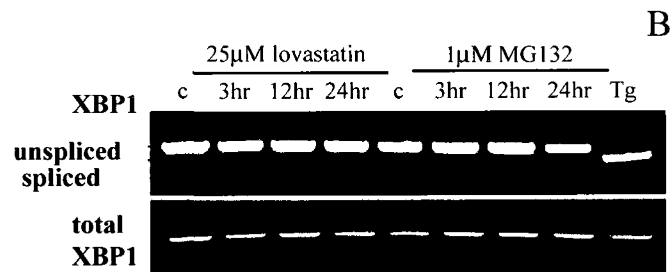
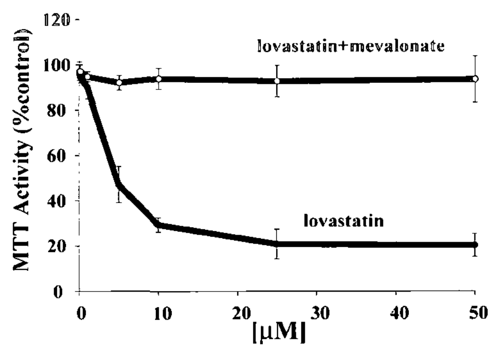
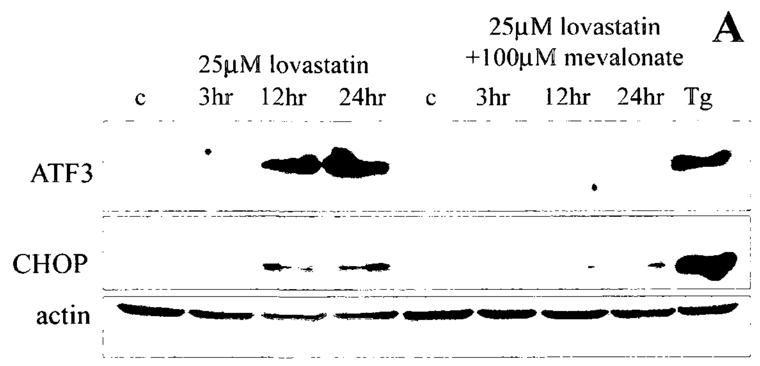
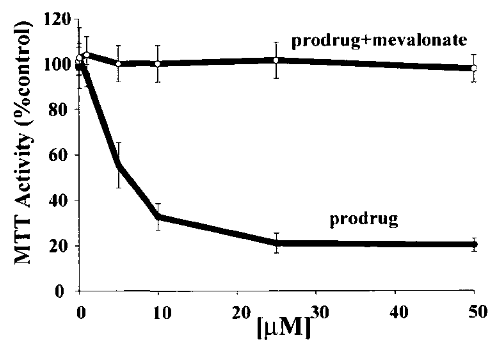
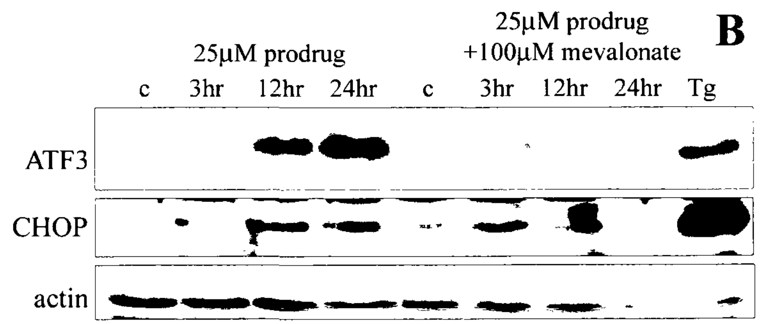

B
25μM lovastatin
1μM MG132
c 3hr 12hr 24hr c 3hr 12hr 24hr Tg
XBP1
unspliced
spliced
total
XBP1

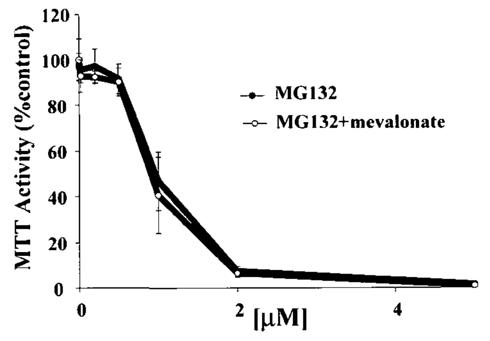
Figure 6. Effect of supplementation with mevalonate on lovastatin and MG132 induced cytotoxicity. MTT viability assays comparing the responses of 0-50μM lovastatin (A), 0-50μM of its prodrug version (B) or 0-5μM of MG132 with or without the co-administration of 100μM mevalonate for 48hrs. Cell viability was assayed with the activity of untreated cells taken to be 100%. Western blot analyses of the integrated stress response regulated proteins ATF3 and CHOP following 25μM lovastatin (A), 25μM of its prodrug version (B) or 1μM of MG132 with or without the co-administration of 100μM mevalonate of SCC25 cells. Cell lysates from 3, 12 and 24hrs treatments as well as solvent control (c) and 1μM Tg treatment for 24hrs were analyzed. Expression levels of actin were assayed as the loading control.


MTT Activity (%control)
lovastatin+mevalonate
lovastatin
[μM]


A
25μM lovastatin
25μM lovastatin
+100μM mevalonate
c 3hr 12hr 24hr c 3hr 12hr 24hr Tg
ATF3
CHOP
actin


MTT Activity (%control)
prodrug+mevalonate
prodrug
[μM]


B
25μM prodrug
25μM prodrug
+100μM mevalonate
c 3hr 12hr 24hr c 3hr 12hr 24hr Tg
ATF3
CHOP
actin


MTT Activity (%control)
MG132
MG132+mevalonate
[μM]

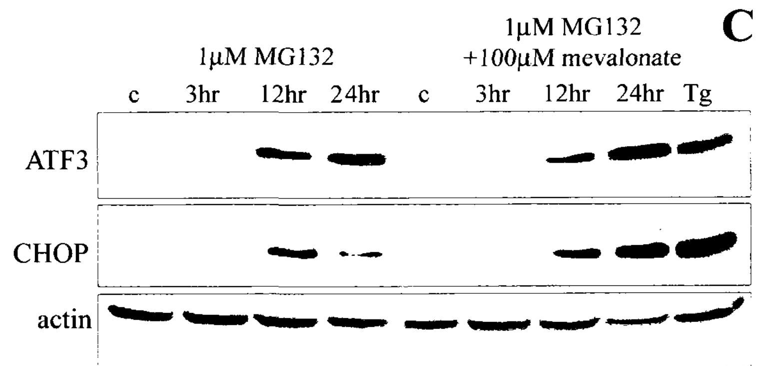
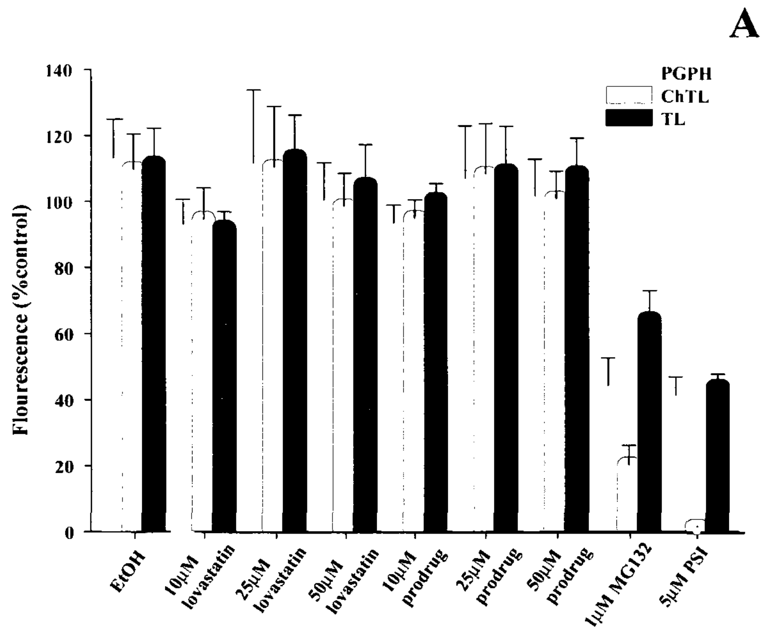
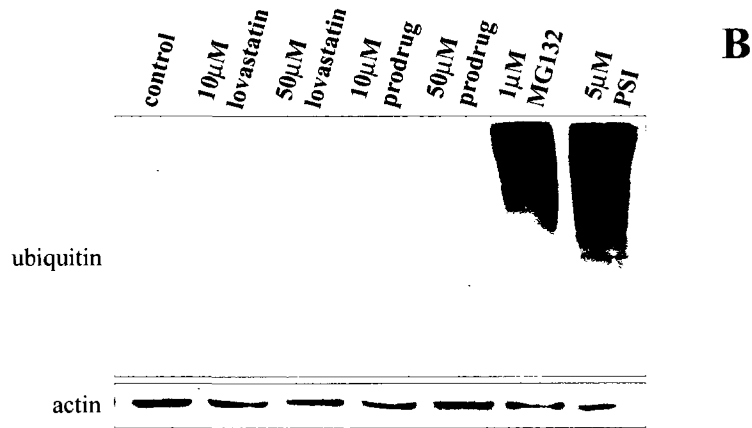
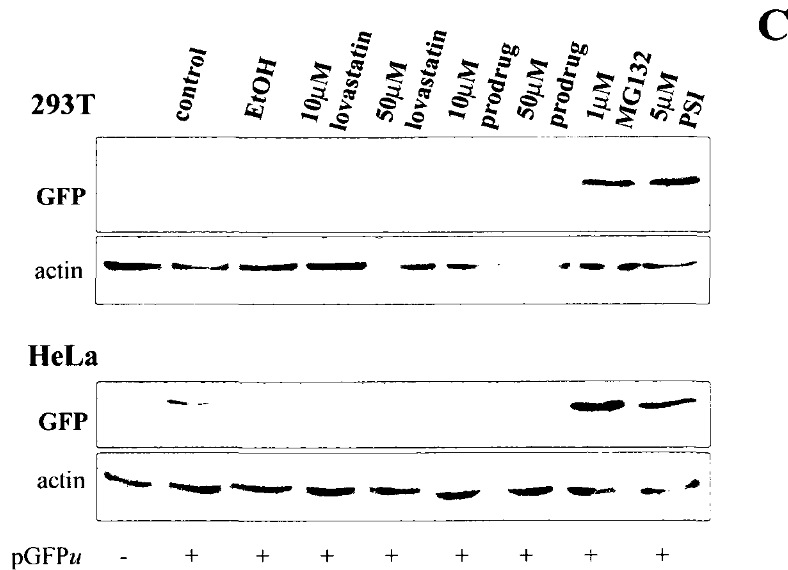

C
1μM MG132
1μM MG132
+100μM mevalonate
c 3hr 12hr 24hr c 3hr 12hr 24hr Tg
ATF3
CHOP
actin

Figure 7. Analysis of proteasome inhibitor activity of lovastatin (drug and prodrug). A, Effect of lovastatin, its prodrug version, MG132 and PSI on the PGPH, chymotrypsin-like (ChTL) and trypsin-like (TL) activity of the SCC25 cell 26S proteasome. For each treatment, proteasome activity was measured using the fluorogenic substrates described in the Experimental Procedures section. The results represent the mean ± SEM of four experiments. B, Western blot analyses of ubiquitin following 10 and 50μM lovastatin, 10 and 50μM of its prodrug version, 1μM of MG132 or 5μM PSI treatments of SCC25 cells. Cell lysates 24hrs treatments as well as solvent control (c) were analyzed. Expression levels of actin were assayed as the loading control. C, Western blot analyses of GFP following transfection of 293T and HeLa cells with a plasmid expressing an unstable GFPu variant that is rapidly degraded by the proteasome. Lysates from 24hrs treatments of 10 and 50μM lovastatin, 10 and 50μM of its prodrug version, 1μM of MG132 or 5μM PSI treatments were analyzed. Expression levels of actin were assayed as the loading control.


A
PGPH
ChTL
TL
Flourescence (%control)
140
120
100
80
60
40
20
0
EtOH
10μM lovastatin
25μM lovastatin
50μM lovastatin
10μM prodrug
25μM prodrug
50μM prodrug
1μM MG132
5μM PSI


B
control
10μM lovastatin
50μM lovastatin
10μM prodrug
50μM prodrug
1μM MG132
5μM PSI
ubiquitin
actin


C
293T
control
EtOH
10μM lovastatin
50μM lovastatin
10μM prodrug
50μM prodrug
1μM MG132
5μM PSI
GFP
actin
HeLa
GFP
actin
pGFPu - + + + + + + + +

DISCUSSION

This study is the first demonstration that lovastatin treatment of HNSCC cells and MEFs induces a potent ISR. Furthermore, we show that the ISR mediates the apoptotic effects of lovastatin in these cells. Lovastatin induced stress displays the molecular characteristics associated with this response. Lovastatin induced the phosphorylation of eIF-2α which inhibited global protein translation but allowed for the selective translational induction of ATF4. Transcriptional targets of ATF4 that were up regulated both at the mRNA and protein levels included ATF3 and CHOP. A transcriptional target of CHOP, GADD34 was then subsequently induced. Lack of CHOP expression in CHOP-/- MEFs attenuated the apoptotic response of lovastatin, a key regulator of ISR induced apoptosis. Taken together, these results clearly indicate the role of the ISR in lovastatin-induced apoptosis.

HMG-CoA reductase and the ISR may be linked due to the potential of mevalonate metabolites to affect cellular homeostasis. First, dolichol is involved in the N-linked glycosylation of proteins within the ER necessary for proper protein maturation [1]. Tunicamycin, a potent inhibitor N-linked glycosylation, induces the ER stress response in a wide variety of cell types [40]. Alterations of membrane cholesterol composition can also trigger the ISR as inhibitors of cholesterol biosynthesis can induce this response [41]. Proteins that require farnesyl and geranylgeranyl modifications include the Rho and Rab families that play critical roles in ER homeostasis [2, 3]. They regulate the actin cytoskeleton and intracellular trafficking and their perturbations have been demonstrated to result in ER stress as well. For example, brefeldin A, an inhibitor of RAB6 function that regulates ER to golgi vesicular trafficking, induces ER stress [42]. Isopentyladenine is a mevalonate metabolite that is a component of some

transfer RNAs critical to protein production [43]. Rab proteins also regulate the internalization and trafficking of amino acid transporters [44]. Ubiquinone is an important component of the mitochondrial respiratory chain and is a powerful antioxidant and a membrane stabilizer [1]. Ubiquinone depletion disrupts ATP synthesis and may lead to oxidative cellular stress. Thus, a number of mevalonate metabolites whose depletion by lovastatin treatment have the potential to trigger cellular stress resulting in apoptosis.

Important inducers of the ISR are a family of protein kinases that phosphorylate eIF2α under stress conditions [14]. Phosphorylation of eIF2α inhibits recycling of eIF2 to its active GTP-bound form inhibiting global translation allowing cells to conserve resources and induce gene expression to enable cell recovery [14]. Four different eIF2α kinases have been identified that recognize a variety of these stress conditions. PERK is activated in response to misfolded protein in the ER and to oxidative stress [15]. GCN2 is induced during amino acid deprivation by a mechanism that involves uncharged tRNA binding to a regulatory region found within this enzyme [17]. GCN2 is also activated by other stresses including UV irradiation and proteasome inhibition [17]. Additional mammalian eIF2 kinases include PKR which participates in an anti-viral defense mechanism [16] and HRI that is activated by heme deprivation in erythroid tissues [18]. In this study, we demonstrated a role for GCN2 in regulating lovastatin-induced ATF4 induction and cytotoxicity in MEFs. Identifying the upstream GCN2 activating targets of lovastatin may uncover a more relevant and specific therapeutic target.

Regulation of protein degradation plays an important role in controlling cell cycle and apoptotic signaling by affecting the levels of cellular proteins in these pathways [39]. Ubiquitination of many of these proteins through the covalent linkage of ubiquitin, targets their degradation by the ATP dependant 26S proteasome [39]. Proteasome inhibitors can induce

apoptosis in a variety of human tumour cells and clinical evaluations have shown the potential for these inhibitors as therapeutic options [45]. Recently, the apoptotic effects of proteasome inhibitors have been shown to be regulated at least in part by their ability to activate the ISR [23]. This is likely induced by the accumulation of misfolded proteins. In MEFs, GCN2 phosphorylation of eIF2α likely regulates this response [23]. In this study, we also demonstrated the ability of lovastatin to illicit a potent ISR that was regulated by GCN2 in MEFs. Since the prodrug version of lovastatin was previously demonstrated to be an inhibitor of the 26S proteasome [22], we evaluated the role of proteasome inhibition in inducing the ISR in response to lovastatin. While both proteasome inhibitors and lovastatin induce the ISR, it is clear in this study that neither lovastatin nor its prodrug form affects proteasome function in these cells. In a previous study, we demonstrated that simply adding the prodrug form to media could readily transform it to its open active form [10]. Since mevalonate reversed the cytotoxic effects and ISR inducing properties of both forms of lovastatin and that mevalonate itself did not affect proteasome function, conversion of the prodrug to the active form and targeting HMG-CoA reductase is the mechanism regulating these activities. Therefore, lovastatin induces the ISR in HNSCC cells and MEFs through a novel and as yet unrecognized mechanism.

Identifying the mevalonate metabolites that regulate lovastatin-induced apoptosis in HNSCC and other malignant cells may have profound clinical significance. Identifying these mediators may uncover a novel more refined therapeutic approach.

Experimental Procedures

Tissue Culture- The SCC25, HeLa and the 293T cell lines were obtained from the American Type Culture Collection (Rockville, MD). The wild type +/+, CHOP-/- and GCN2-/- murine

embryonic fibroblast cell lines (MEFs) were kindly provided by Dr. David Ron (Skirball Institute, New York) while the PERK-/- MEFS were provided by Dr. John Bell (Ottawa Health Research Institute, Ottawa). These cell lines were maintained in Dulbecco's-MEM (Media Services, Ottawa Regional Cancer Centre) supplemented with 10% fetal bovine serum (Medicorp, Montreal). Cells were exposed to solvent control or to 0-100μM lovastatin (generously provided by Apotex, Mississauga, Canada, diluted from a 10mM stock in ethanol prepared as previously described[24]). Thapsigargin (Tg) was purchased from Sigma (St. Louis, MI).

Western Blot Analysis- Total cellular protein was extracted using a buffer that consisted of 1% Igepal CA-630 (Sigma), 0.5% sodium deoxycholate (Sigma), 0.1% SDS (Sigma), 0.2mM sodium orthovanadate (Sigma) and 0.2mM phenyl methyl sulphonyl fluoride (Sigma) in 2xPBS. Approximately 200μl of extraction buffer was used to treat 10^6 cells. Total protein was quantified with the Biorad Protein Assay using bovine serum albumin (Sigma) as standard. Protein extracts representing 50μg total protein were separated on a 10% SDS-PAGE gel and electrophoretically transferred onto PVDF membranes (Amersham, Toronto, ON). Membranes were blocked in 5% skim milk powder in PBS overnight at 4^0C. Primary antibody, diluted in 5% skim milk powder in PBS, was incubated with the membrane for 1hr at room temperature. The antibodies used in this study included ATF4, ATF3, CHOP, GADD34, ubiquitin and green fluorescent protein (GFP) (Santa Cruz Biotechnology, Santa Cruz, CA), BiP, eIF2α and HSP70 (Stressgen, Ann Arbor, MI) and phosphoserine 51 eIF2α (Cell Signaling, Danvers, MA). The secondary antibodies (Amersham) were applied at a 1:5000 dilution in 3% BSA, 10% FBS in PBS and incubated for 1hr at room temperature (washes following antibody incubations are 3x5min in PBS/0.05% Tween 80 (Sigma) then processed for chemiluminescent

detection (Amersham). After the desired exposure was obtained the membrane was stained with Coomassie Blue (Sigma) to ensure equal loading of the samples. For visualizing exogenous GFP levels, the pEGFP-C1 plasmid with a degron domain added to the C-terminus (GFPu) [25] provided by Dr. Doug Gray (Ottawa Health Research Institute, Ottawa) was transfected into HeLa and 293T cells using FuGENE transfection reagent (Roche, Mississauga, ON) following manufacturer's instructions.

Real time RT-PCR-mRNA expression was measured using quantitative RT-PCR. cDNA was prepared from total RNA extracted from cells using Superscript II reverse transcriptase and p(dT) primer according to the manufacturer's protocol (Invitrogen, San Diego, CA). Transcripts were amplified in 20µl reactions containing 1X PCR buffer (20mM Tris-HCl, pH 8.4, 50mM KCl), 100uM dNTPs, 200µM gene specific primers, 100ng cDNA template, 0.02U/µl Platinum *Taq* DNA polymerase (Invitrogen) and Sybr green I (Molecular Probes, Carlsbad, CA). Amplification was carried out on a LightCycler thermocyler (Roche) using 50 cycles of 95°C for 20s, 58°C for 10s, 72°C for 24s. Fold changes, expressed as means (+/- SEM, n=3), were calculated for drug treated groups versus vehicle control using ΔΔCt values following normalization to *GAPDH* transcript levels. The primers for gene-specific RT-PCR analysis were as follows: *ATF3*: TAGGCTGGAAGAGCCAAAG(5') and TTCTCACAGCTGCAAACACC(3'); *ATF4*: AGTGATATCCACTTCACTGCCCAG(5') and AAGGAGTTCGCCTTGGATGCCC TG(3'); *CHOP*: CTCTGACTGGAATCTGG AGAGTG(5') and CTGAGTCATTGC CTTTCTCTTCG(3'); *GAPDH* TTGATG TCATCATACTTGGCAGGT(5')and CAG TCAAGGCTGAGAATGGGA(3').

RT-PCR- XBP-1 cDNAs were prepared as described above. The primers and amplification parameters to detect splicing of *XBP-1* were previously described [26] and were as follows: spliced version CCTTGTAGTTGAGAACCAGG (5'), unspliced version CAGCAGGTGCAGGCCCAGTT (5') and *XBP-1* total GGGGCTTGGTATATATGTGGG (3'). The PCR products were electrophoresed through a 2% agarose gel and visualized by ethidium bromide staining and documented using Gene Genius BioImaging System (Syngene, Frederick, MD).

S^{35}Radiolabeling Assays- Treated SCC25 cells in Dulbecco's modified Eagle's medium supplemented with 10% FBS were washed in PBS followed by 30 min incubation in translation labeling medium without methionine (Invitrogen). SCC25 cells were labeled with 20μCi/ml [^{35}S]Met (contents) for 15 min then washed three times with ice-cold PBS. Cell lysates were prepared and equal amounts of protein were separated by SDS-PAGE and radiolabeled proteins were visualized by autoradiography.

MTT Assay- In a 96 well flat bottom plate (Nunc, Naperville, Il.) approximately 5,000 cells/150μl of cell suspension was used to seed each well. The cells were incubated overnight to allow for cell attachment and recovery. Following treatment, 50μl of a 5mg/ml solution in phosphate buffered saline of the MTT tetrazolium substrate (Sigma) was added and incubated for up to 6 hrs at 37°C. The resulting violet formazan precipitate was solubilised by the addition of 100μl of a 0.01M HCl/10% SDS (Sigma) solution shaking overnight at 37°C. The plates were then analyzed on an MRX Microplate Reader from Dynex Technologies at 570 nm to determine the optical density of the samples.

Flow Cytometry- Cell cycle parameters were determined by flow cytometry using propidium iodide labeling of single cells as described previously [10]. Single cell suspensions were labeled with 50μg/ml propidium iodide (Sigma) and approximately 10^6 cells in 1ml analyzed by flow cytometry. Ten thousand cells were evaluated and the percentage of cells in subG1 phase was determined using the Modfit LT program (Verity Software House, Topsham, Maine).

Fluorogenic Proteasome Assay- The fluorogenic substrates used in this study were as follows: Z-Leu-Leu-Glu-AMC (7- Amino-4-methylcoumarin) (PGPH activity; Calbiochem, Mississauga, ON), Suc-Leu-Leu-Val-Tyr-AMC (ChTL activity; Calbiochem), and Bz-Val-Gly-Arg-AMC (TL activity; BioMol, Plymouth Meeting, PA). SCC25 cells were treated with lovastatin, its prodrug form, MG132 (carbobenzoyl-L-leucyl-L-leucyl-L-leucinal, Calbiochem) and PSI (Z-ile-Glu(OtBu)-Ala-leu-CHO, Calbiochem). Treated cells were washed in ice cold Buffer I (50mM Tris-HCl pH 7.5, 1mM DTT, 5mM $MgCl_2$, plus 2mM fresh ATP) then scraped in lysis buffer consisting of 50mM Tris-HCl pH7.5, 1mM DTT, 5mM $MgCl_2$, 250mM sucrose, and 2mM ATP. Lysates were vortexed for 1min with glass beads (<106μm acid washed; Sigma) to disrupt cells. The beads were subsequently removed by a 5min centrifugation at 1500g, then cellular debris removed by a 10min spin at 10,000g. Total protein was quantified by the BCA method (Pierce, Rockford, IL), and 10μg protein diluted to 50μL with Buffer I. Each sample was then combined with 50 μL of Buffer I containing diluted substrate such that the final concentration of fluorogenic substrate was 50μM (ChTL) or 100μM (PGPH and TL). Samples were incubated with substrates for approximately 30min in the dark then fluorescence of the cleaved AMC molecule measured at 460nm using a Fluoroskan Ascent FL plate reader (Thermo Labsystems,

Nepean, ON) . Each sample was assayed in replicates of five to derive a mean value and the data shown represent the average of the means from at least 3 independent experiments.

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CHAPTER 3

THE INTEGRATED STRESS RESPONSE IS DIFFERENTIALLY INDUCED IN LOVASTATIN TREATED TUMOUR CELLS: ENHANCED CYTOTOXICITY WITH SALUBRINAL *

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Running head: ISR regulates lovastatin-induced apoptosis
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Abstract

We have previously demonstrated the ability of lovastatin, a potent inhibitor of mevalonate synthesis, to induce tumour specific apoptosis. In our recent study, the apoptotic effects of lovastatin were regulated in part by the integrated stress response (ISR). The ISR plays a significant role in regulating cellular responses to a wide variety of stress inducers either alleviating the stress or alternatively inducing apoptosis. Phosphorylation of the eukaryotic initiation factor 2 (eIF2α) by the ISR inhibits protein translation but enhances the expression of the activating transcription factors (ATF) 4, ATF3 and their target gene *CHOP* that can induce apoptosis. In this study, we show that squamous cell carcinoma (SCC) derived cell line SCC25 and HeLa cells, sensitive to lovastatin induced apoptosis, preferentially induced the ISR compared to resistant breast and prostate carcinoma derived cell lines. In HeLa cells, shRNA targeting ATF3 expression, as well as in murine embryonic fibroblasts (MEFs) deficient in ATF3 expression, lovastatin induced cytotoxicity and apoptosis were attenuated. Over-expression of the phosphorylation-insensitive s51a mutant of eIF2α in MEFs inhibited lovastatin-induced cell death. Moreover, we report that using salubrinal, an inhibitor of PP1, the phosphatase responsible for dephosphorylation of eIF2α, further increased the expression of ATF3 and enhanced lovastatin induced cell death in SCC25 cells and MEFs. Taken together, our results demonstrate differential activation of the ISR in lovastatin sensitive tumour derived cell lines and in combination with salubrinal enhanced cytotoxicity is demonstrated suggesting a novel combinational therapeutic strategy.

Introduction

Statins are potent inhibitors of HMG-CoA reductase, the rate limiting enzyme of the mevalonate pathway, that are widely used as hypercholesterolemia treatments (1). Mevalonate pathway metabolites are a critical requirement for the function or the appropriate expression/localization of a wide array of signaling proteins (2,3). The rate-limiting step of the mevalonate pathway is the conversion of HMG-CoA to mevalonate, which is catalyzed by HMG-CoA reductase (3). The mevalonate pathway produces various end products that are critical for many different cellular functions. These products include cholesterol, dolichol, geranylgeranyl pyrophosphate (GGPP), and farnesyl pyrophosphate (FPP). Cholesterol is essential in maintaining cellular membrane structure and integrity. Dolichol is a carrier molecule of oligosaccharides in N-linked protein glycosylation for the production of glycoproteins. Geranylgeranyl transferase and farnesyl transferase use GGPP and FPP, respectively, for post-translational modifications of a wide variety of cellular protein (2-4). These include Ras and many small GTP-binding proteins such as members of the Rab, Rac, and Rho families. These proteins regulate cell proliferation, cell survival, intracellular trafficking and cell motility and this post-translational modification functions as a membrane anchor critical for their cellular localization and activity (2,4). Blockade of the rate-limiting step of the mevalonate pathway by HMG-CoA reductase inhibitors results in decreased levels of mevalonate and its downstream products and, thus, has significant influences on many critical cellular signaling pathways.

Malignant cells appear highly dependent on the sustained availability of the end products of the mevalonate pathway (5,6). Deregulated or elevated activity of HMG-CoA reductase has been shown in a range of different tumours. The statin family of drugs are potent inhibitors of HMG-CoA reductase that bind to the active site of HMG-CoA reductase with up to a 1000x

higher affinity than its natural substrate (1). Recent analyses have demonstrated that statin treatment can directly block tumour cell growth, invasion and metastatic potential both *in vitro* and *in vivo* (6-9). Because HMG-CoA reductase inhibitors exhibit such diverse effects on tumour cells, numerous clinical trials were undertaken to assess potential clinical benefit, however, no significant anti-tumour responses were observed (10). New optimism regarding the use of HMG CoA reductase inhibitors as anti-neoplastic agents has emerged from recent studies. We have previously shown that lovastatin can induce tumour specific apoptosis especially in squamous cell carcinomas (SCC) (11,12) and that 23% of SCC of the head and neck and cervix patients treated with lovastatin as a single agent showed disease stabilization in our Phase I clinical trial (13). These results suggest that clinical utility of statins would likely be in combination based therapeutic approaches. Understanding the mechanism of lovastatin-induced apoptosis may uncover such novel therapeutic strategies.

To this end, our recent studies have identified a previously unappreciated cellular signaling pathway that is targeted by lovastatin treatment and also regulates lovastatin induced apoptosis. We have demonstrated the induction of the integrated stress response (ISR) as a key mediator of lovastatin induced apoptosis (14). A variety of cell stresses lead to the phosphorylation of eukaryotic initiation factor (eIF) 2α shutting down global protein translation to potentially alleviate the stress (15-17). The α subunit of eIF2 is the target of a family of serine or threonine kinases that are activated by different forms of environmental stress and include PKR, PERK, GCN2 and HRI (17). For example, PKR senses viral infection, PERK detects endoplasmic reticulum (ER) stress and oxidative stress, GCN2 senses amino acid starvation and HRI monitors changes in the availability of heme during erythrocyte differentiation (17). Each of these stress-activated kinases phosphorylates eIF2α on serine 51, a modification that prevents the

assembly of the pre-initiation complex, and halts global protein translation (17). Under these conditions, however, the translation of activating transcription factor (ATF) 4 is enhanced and plays a significant role in modulating cellular responses to stress (18).

We have identified ATF4 as a lovastatin induced gene in SCC cells (19). Depending on the strength of the stress stimulus, ISR induction can either alleviate the stress or alternatively induce apoptosis. Enhanced expression of the transcription factors *ATF3* and *CHOP*, ATF4 target genes, can mediate this apoptotic response (16,17,20). We have demonstrated that lovastatin treatment induced eIF2α phosphorylation and inhibited global protein translation in SCC cells (14). ATF4 expression was induced followed by increased ATF3 and CHOP expression, targets of ATF4 activity, in SCC25 cells. In CHOP-/- murine embryonic fibroblasts (MEFs), lovastatin-induced apoptosis was attenuated indicating a role for CHOP and the ISR in this response (14). This was the first study to demonstrate that lovastatin, by targeting mevalonate synthesis, is a potent inducer of the ISR.

In this report we evaluated the potential of lovastatin to induce the ISR in apoptosis sensitive and resistant tumour derived cell lines. We further characterized the role of the ISR as a mediator of lovastatin-induced apoptosis. Of significance, inhibition of eIF2α dephosphorylation by salubrinal, enhanced both lovastatin-induced ATF3 expression and apoptosis of SCC cells.

RESULTS

Differential Induction of the ISR by Lovastatin

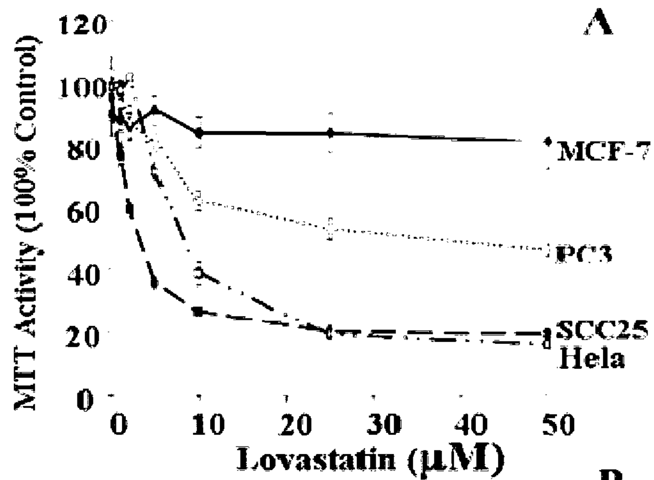
In this study, we evaluated the induction of the ISR in four tumour derived cell lines that we previously demonstrated were either relatively resistant or sensitive to lovastatin-induced apoptosis at clinically relevant doses. The MCF-7 breast adenocarcinoma and the PC3 prostate carcinoma cell lines are resistant while the SCC derived cell lines SCC25 and HeLa are sensitive to lovastatin treatment induced cytotoxicity (12). Employing the MTT cell viability assay and flow cytometric analysis of DNA content, we confirmed the differential cytotoxic and apoptotic effects of lovastatin in these cell lines, respectively (Figure 1). The MTT assay determined the lethal dose (LD) 50, a dose where MTT activity is reduced by 50%, of less than 10μM for the SCC25 and HeLa cell lines, while for MCF-7 and PC3 the LD50 was greater than 40μM lovastatin treatment for 48hrs (Figure 1A). Using flow cytometry to measure cellular DNA content, we demonstrated that while MCF-7 cells treated with 10 and 25μM lovastatin for 48hrs did not display a significant apoptotic response as a characteristic preG1 apoptotic peak (21) was not evident. However, in HeLa cells with identical treatments, a pronounced preG1 apoptotic peak representing the majority of cells was evident (Figure 1B). A hallmark of pre-apoptotic cells is mitochondrial fragmentation (22). Cytochrome c localization, a resident mitochondrial protein that is released in late stages of apoptosis, is employed to visualize mitochondrial structure (23). Untreated SCC25 cells display a tubular mitochondrial network while 10μM lovastatin treated cells for 24hrs show fragmented mitochondria (Figure 1C). These results highlight the differential sensitivity of tumour cells to lovastatin-induced apoptosis.

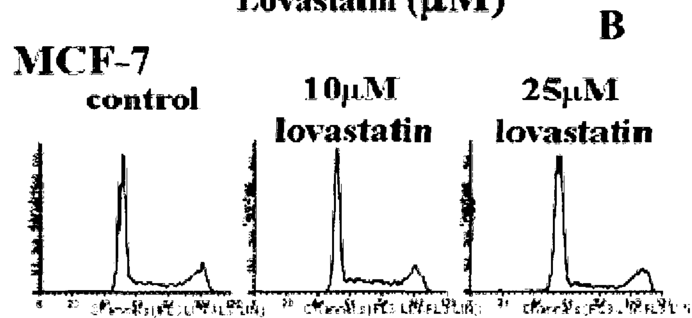
In a previous study, we demonstrated that the ISR was induced by lovastatin in SCC25 cells through the activation of the GCN2 kinase (14). This resulted in phosphorylation of eIF2α,

global protein translation attenuation, and enhanced expression of ATF4, ATF3 and CHOP, transcription factors that trigger expression of a wide variety of factors that can either alleviate the stress or induce apoptosis (16,17,20). Lovastatin-induced apoptosis was also attenuated in GCN2-/- and CHOP-/- MEFs, clearly indicating a role for the ISR in regulating lovastatin-induced apoptosis (14). In this study, we compared the induction of the ISR mediators ATF3, ATF4 and CHOP by lovastatin in the resistant and sensitive tumour cell lines described above. Using quantitative RT-PCR, 24hr lovastatin treatments from 1-50μM did not induce ATF3, ATF4 or CHOP mRNA expression in MCF-7 cells. In contrast, treatment of HeLa cells readily induced ATF3, ATF4 and CHOP levels in a dose dependant manner (Figure 2A).

We next examined the protein expression levels of ATF3 following lovastatin treatment in the above four cell lines to determine a possible association with ATF3 induction and their sensitivity to lovastatin induced-apoptosis. ATF3 is of significance in not only the ISR pathway but in tumourigenesis as well as it has been implicated as both an oncogene and a tumour suppressor gene depending on cellular context (24,25). In this study, 1μM TG treatment was used as a control for the induction of the ISR as it induces ER stress through calcium release (26). In all four cell lines under study, TG induced ATF3 indicating that the ISR is functional. In the MCF-7 and PC3 cell lines, 24hr lovastatin treatment (1-50μM) failed to induce ATF3 expression with only a weak induction in the 50μM treatment of PC3 cells (Figure 2B). In the SCC derived cell lines, ATF3 was significantly induced in a dose dependent manner at levels that were similar to the TG treatments (Figure 2B). Taken together, these data suggests an association between activation of ISR and induction of apoptosis by lovastatin in these tumour derived cell lines.

Figure 1. Lovastatin selectively induced apoptosis in SCC cells compared to breast and prostate carcinoma derived cell lines. A, MTT cell viability assay comparing the response of HeLa, SCC25, PC-3 and MCF-7 cells to lovastatin treatment. Cells were treated with 0–50 μM lovastatin for 48 h, and cell viability was assessed with the activity of untreated cells taken to be 100%. B, Flow cytometric analysis of subG1 apoptotic fraction as determined by propidium iodide staining of cellular DNA content. HeLa and MCF7 cells were treated with 10 and 25μM lovastatin for 48 hr, fixed, then stained with propidium iodide and analyzed by flow cytometry. Significant differences in sub-G1 apoptotic cell are clearly visible. C, SCC25 cells were incubated in the presence of vehicle or 10μM lovastatin for 24hr followed by immunostaining for cytochrome c. Mitochondrial fragmentation is present in lovastatin treated cells


A
MTT Activity (100% Control)
120
100
80
60
40
20
0
MCF-7
PC3
SCC25
Hela
0
10
20
30
40
50
Lovastatin (µM)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MCF-7
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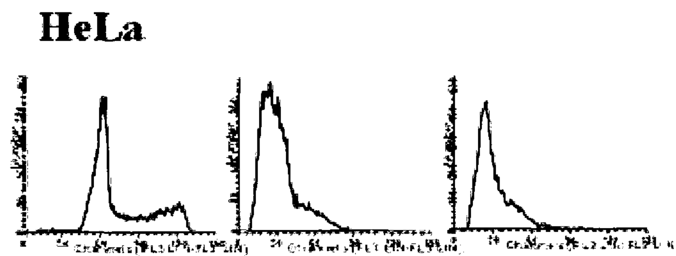

HeLa

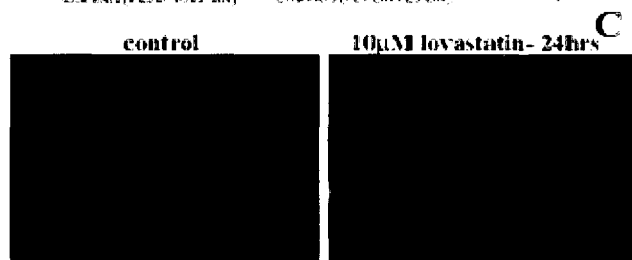
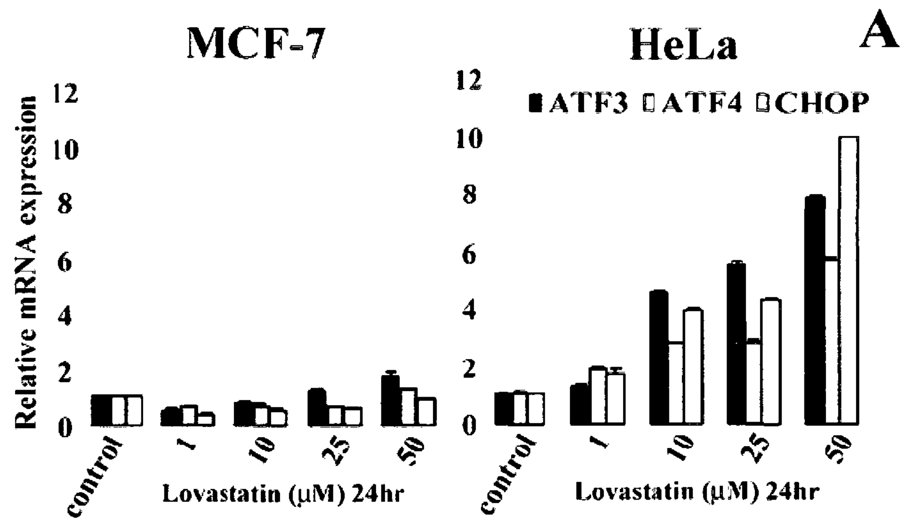
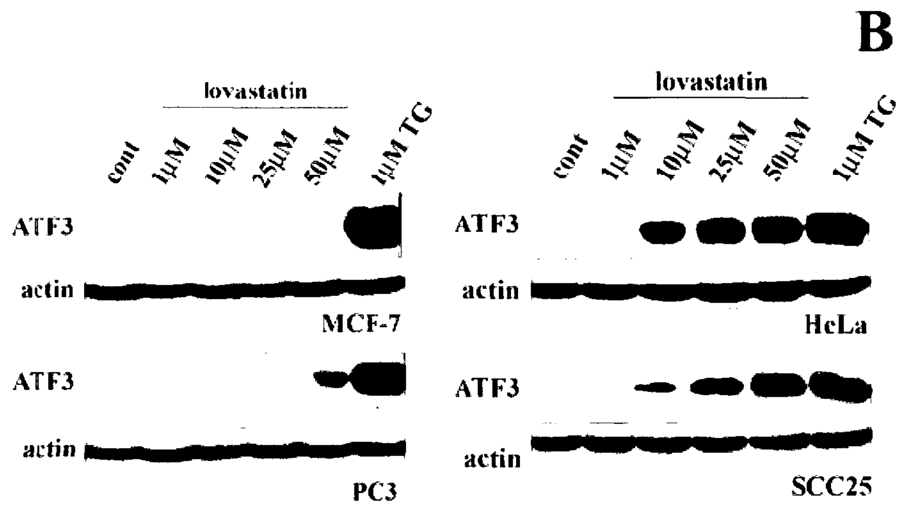

C
control
10µM lovastatin- 24hrs

Figure 2. Lovastatin selectively induces ISR in sensitive cell lines. A, Levels of *ATF3, ATF4* and *CHOP* mRNA were analyzed by real time quantitative RT-PCR following lovastatin treatment in HeLa and MCF-7 cells. Total RNA extracted from 1, 10, 25 and 50 μM lovastatin treatments for 24hrs as well as solvent control were analyzed. Fold changes were calculated following normalization to *GAPDH* levels (ΔΔCt) and expressed as means (±S.E.) (n = 3). B, Immunoblot analysis of ATF3 protein levels in HeLa, PC-3, SCC25 and MCF-7 cells following lovastatin treatment. Cell lysates were prepared from cells treated with 1, 10, 25 and 50μM lovastatin for 24hrs as well as solvent control and 1μM thapsigargin. Expression levels of actin were assayed as the loading control.



MCF-7
HeLa
A
ATF3 ATF4 CHOP
Relative mRNA expression
12
10
8
6
4
2
0
control
1
10
25
50
Lovastatin (μM) 24hr



B
lovastatin
cont
1μM
10μM
25μM
50μM
1μM TG
ATF3
actin
MCF-7
HeLa
PC3
SCC25

ATF3 Regulates Lovastatin-Induced Apoptosis

Besides the ISR, activation of ATF3 by a wide array of stress signaling pathways has been demonstrated including DNA repair pathway components such as p53 and the stress induced MAP kinase cascades that include the SAPK/JNK, p38 and ERK pathways (24,25). Previously, we had shown that activation of ISR by lovastatin is mediated through a GCN2-ATF4 dependent pathway (14). In this study, we sought to determine whether induction of ATF3 by lovastatin is solely dependent on the ISR as well as the role of ATF3 in regulating lovastatin-induced apoptosis. MEFs generated from murine knockout models deficient in ATF4 and GCN2 were employed to evaluate the role of the ISR in lovastatin-induced ATF3 expression. Using Western blot analysis, ATF4+/- MEFs readily induced ATF3 following 24hr lovastatin treatments of up to 25μM (Figure 3A). In MEFs deficient in ATF4 or GCN2, similar lovastatin treatments failed to induce ATF3 expression (Figure 3A). Furthermore, in GCN2-/- MEFs phosphorylation of its downstream target eIF2α was only detected following TG treatment and not with lovastatin treatment or with treatment of the proteasome inhibitor MG132 that has been reported to induce the ISR through GCN2 activation (Figure 3B) (27). In wild type MEFs, similar to lovastatin, TG and MG132 treatments induced p-eIF2a as previously shown (14) (Supplemental Figure 1). Activation of ISR through a GCN2/eIF2α/ATF4 pathway regulates ATF3 induction in apoptotic sensitive cells.

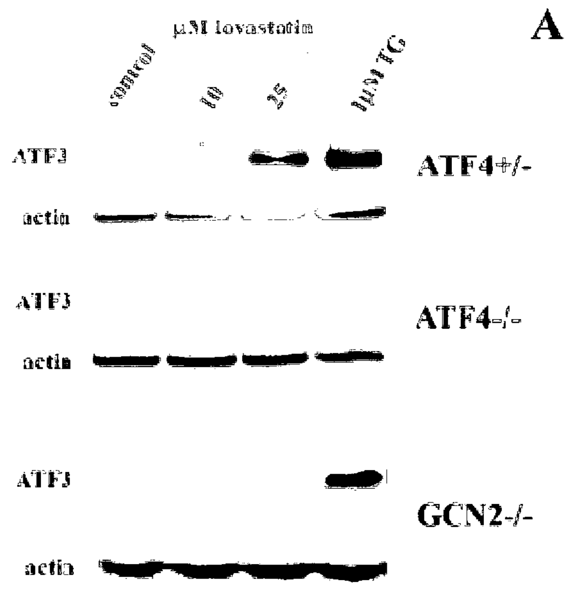
To test the role of ATF3 in regulating lovastatin-induced apoptosis, we evaluated MEFs deficient in ATF3 and shRNA expressing HeLa cells that target *ATF3* expression. Western blot analysis showed that the induction of ATF3 by lovastatin in wild-type MEFs was associated with the cleavage of PARP, where the 85kD cleavage product was visualized and is a characteristic of apoptotic cell death (28). However, in ATF3-/- MEFs similar lovastatin- treatments of up to

25μM for 24hrs did not lead to significant cleavage of PARP (Figure 4A). To further characterize the role of ATF3 in lovastatin induced cytotoxicity, we employed the MTT cell viability assay to evaluate the effects of 0-50μM treatments for 48hrs in ATF3+/+ and ATF3-/- MEFs and two sequence independent shRNAs targeting *ATF3* and green fluorescent protein (*GFP*) expressing HeLa cells. In the ATF3-/- MEFs compared to their wild type (ATF3+/+) counterparts, lovastatin-induced cytotoxicity was attenuated by approximately 2 fold as the LD50 for the ATF3-/- was 12μM and the wt MEFs was 23μM (Figure 4B). Similar results were demonstrated in the HeLa cells expressing the two shRNAs targeting *ATF3* expression in comparison to the shRNA targeting *GFP*. Since ATF3 is not readily detected under normal cell culture conditions (24,29), the shRNA targeting ATF3 were selected based on their ability to inhibit TG (1μM, 24hrs) induced ATF3 expression (Figure 4C). Similar attenuation of lovastatin-induced cytotoxicity was also demonstrated in sh*ATF3* expressing lung carcinoma cell line A549 (Supplemental Figure 2).

A Role for CHOP and Calcium Efflux in Lovastatin-Induced Apoptosis

Following activation of the ISR, ATF3 acts as an upstream activator of CHOP, a pro-apoptotic transcription factor that can regulate the apoptotic effects of the ISR (30). In our previous study, we had shown that CHOP is a mediator of lovastatin-induced apoptosis (14). We evaluated whether the induction of CHOP by lovastatin is mediated through expression of ATF3. To this end, we treated ATF3+/+ and ATF3-/- MEFs with 10μM lovastatin for various

Figure 3. Induction of ATF3 by lovastatin requires ATF4 and GCN2 expression. A, Immunoblot blot analysis of ATF3 protein levels in ATF4+/-, ATF4-/- and GCN2-/- MEFs following lovastatin or thapsigargin (TG) treatment. Cell lysates prepared following 10 and 25μM treatments for 24hrs or 1μM TG for 24hr as well as solvent control were analyzed. B, Immunoblot analysis of p-eIF2α protein levels following lovastatin treatment of GCN2-/- MEFS. Cell lysates from 10 μM lovastatin treatments for 24hr as well as solvent control, 1μM TG for 24hrs or MG132 treatment for 6hrs were analyzed. Expression levels of total eIF2α were assayed as the loading control.


A
μM lovastatin
control
10
25
1μM TG
ATF3
actin
ATF4+/-
ATF3
actin
ATF4-/-
ATF3
actin
GCN2-/-

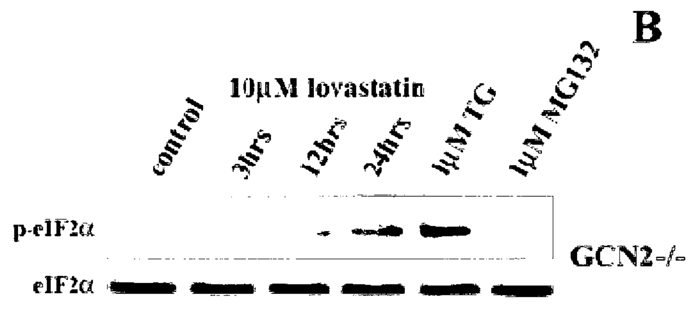
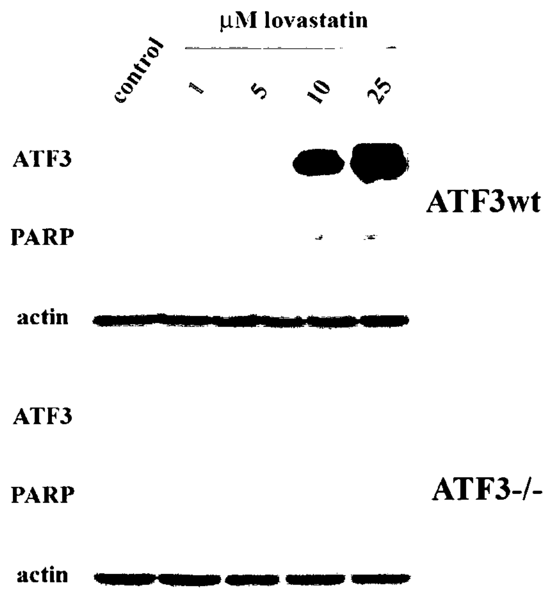
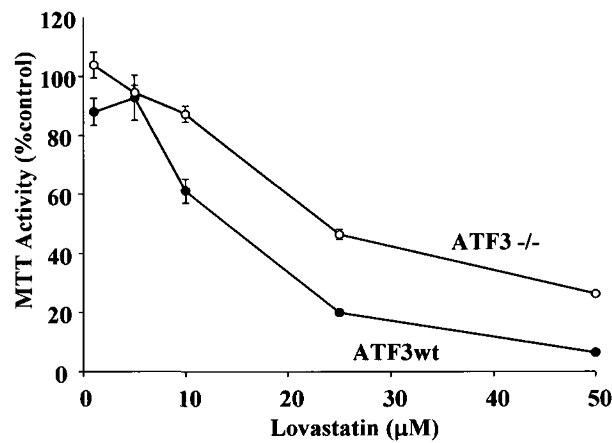

B
10μM lovastatin
control
3hrs
12hrs
24hrs
1μM TG
1μM MG132
p-eIF2α
eIF2α
GCN2-/-

Figure 4. ATF3 regulates lovastatin induced cell cytotoxicity. A, Western blot analysis of ATF3 protein and cleaved PARP levels following lovastatin treatment of ATF3+/+ and ATF3-/- MEFs. Cell lysates from 1, 5, 10 and 25 μM lovastatin treatments for 24hrs as well as solvent control (were analyzed. Expression levels of actin were assayed as the loading control. B, MTT cell viability assays comparing the responses of ATF3+/+ and ATF3-/- MEFs treated with 0–50 μM lovastatin for 48 h, with activity of untreated cells taken to be 100%. C, MTT cell viability assays comparing the responses of HeLa cells stably cloned with short hairpin RNA expressing two different anti-sense sequences against *ATF3* as well as *GFP* as control. Cells were treated with 0–50 μM lovastatin for 48 hr. Immunoblot analysis was used to assess the efficiency of sh-*ATF3* clones for knockdown of ATF3 expression in cells, were treated with 1μM TG for 24hrs, or solvent only as control. Expression levels of actin were assayed as the loading control.



A



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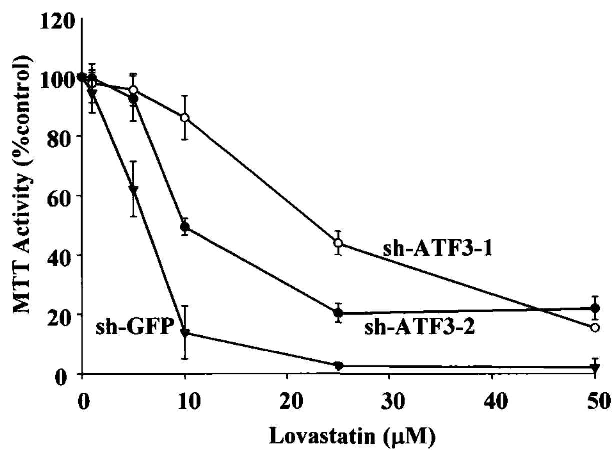
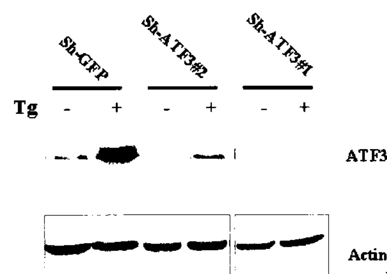




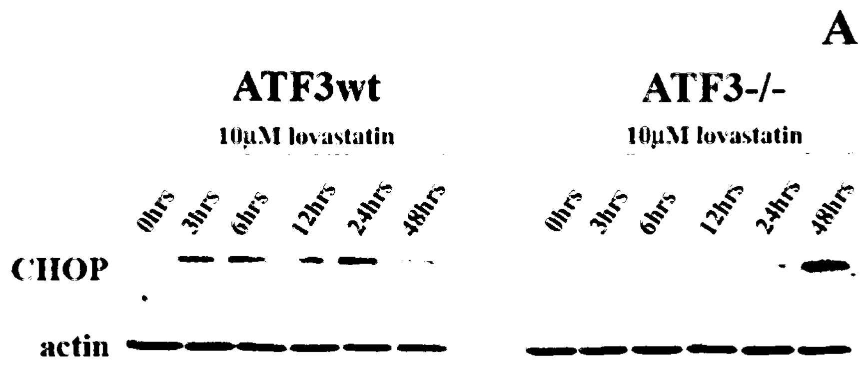
C

time periods up to 48hrs. Induction of CHOP in wt MEFs was detectable as early as 3hrs post-treatment and was sustained for 24hrs with reduced expression at 48hrs, probably due to cell damage. In ATF3-/- MEFs, CHOP expression was not detected until after 24hr treatment at relatively weak levels (Figure 5A). Furthermore, ATF3-/- MEFs treated with 10μM lovastatin for 24hrs, using immunofluorescent microscopy failed to demonstrate expression of significant levels of CHOP in their nuclei. In ATF3+/+ MEFs elevated expression of CHOP in cell nuclei was evident (Figure 5B). These findings support the role of ATF3 as a regulator of lovastatin-induced apoptosis.

CHOP as a transcription factor can regulate among its many targets the expression of various BCL-2 family members that regulate apoptosis through affecting mitochondrial function and intracellular calcium levels (31). In this study, SCC25 cells treated with lovastatin for 24hrs were evaluated for changes in intracellular calcium concentration coincident with treatment. Flow cytometric analysis of SCC25 cells using the Oregon Green 488 BAPTA-AM that fluoresces when bound to intracellular calcium also demonstrated that 24hr 10 and 50μM lovastatin treated SCC25 cells showed an increase in intracellular calcium levels at levels similar to TG treatment (Figure 6A).

In MEFs, Caspase 12 cleavage is often triggered by ISR activators that affect calcium homeostasis (32). Activation of Caspase 12 by its cleavage is regulated in part by the calcium dependent proteolytic calpain enzymes in murine cells (33). In MEFs, lovastatin treatment induced Caspase 12 cleavage in a dose (10 and 50μM) and time dependant (up to 24hrs) manner (Figure 6B). The MTT cell viability assay was used and showed that in MEFs, the co-administration of 30μM calpeptin, a chemical inhibitor of calpains (33), attenuated the cytotoxicity of 48hr treatments of up to 50μM lovastatin (Figure 6C).

Figure 5. Lovastatin induction of CHOP and its nuclear translocation is ATF3 dependent. A, Immunoblot analysis of CHOP protein levels following lovastatin treatment of ATF3+/+ and ATF3-/- MEFS. Cell lysates from 10μM treatments for 0, 3, 6, 12, 24 and 48 hr were analyzed. Expression levels of actin were assayed as the loading control. B, Immunflourescence analysis of CHOP protein (red) following lovastatin treatment of ATF3+/+ and ATF3-/- MEFS, nuclei were co-stained with DAPI (blue).


A
ATF3wt
10μM lovastatin
ATF3-/-
10μM lovastatin
0hrs
3hrs
6hrs
12hrs
24hrs
48hrs
CHOP
actin

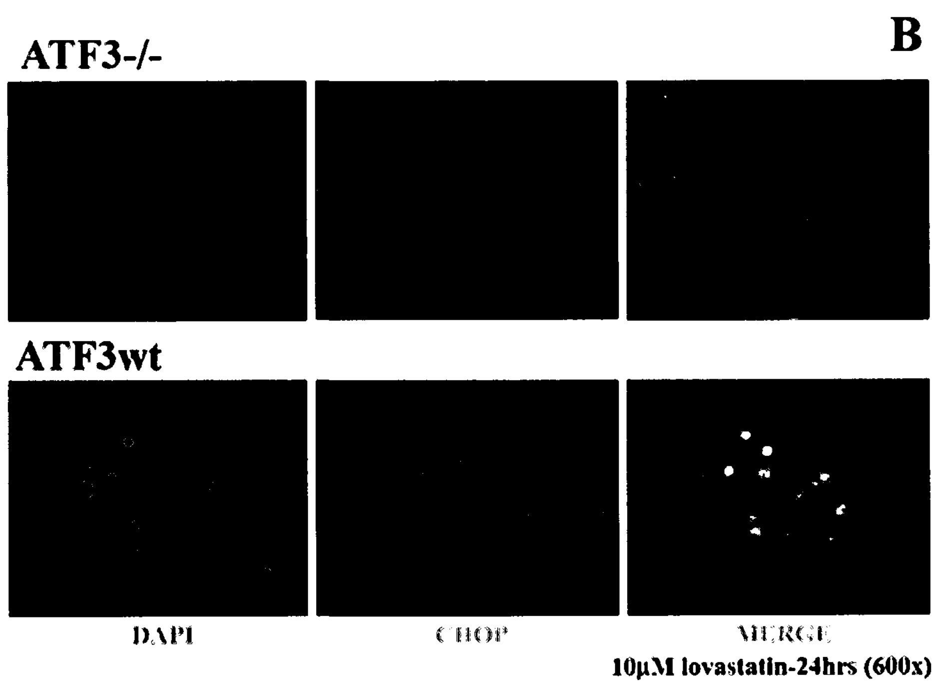
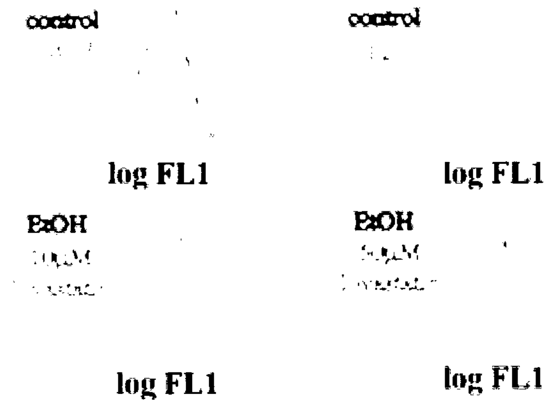

B
ATF3-/-
ATF3wt
DAPI
CHOP
MERGE
10μM lovastatin-24hrs (600x)

Figure 6. Calpeptin inhibits lovastatin induced apoptosis. A, Measurement of intracellular calcium levels following lovastatin treatment in SCC25 cells. Cells exposed to vehicle control (EtOH), or 10 and 50uM lovastatin were loaded with Oregon Green 488 BAPTA-AM then analyzed by FACS at 525nm as described. 1μM TG treatments were carried out on pre-loaded cells for 15min prior to FACS analysis. B, Immunoblot analysis of pro-caspase 12 protein levels following lovastatin treatment of wild-type MEFs. Cell lysates from 10 and 50μM lovastatin treatments for 3, 12 and 24 or 24h or 1μM TG for 24hr as well as solvent control (*c*) were analyzed. Expression levels of actin were assayed as the loading control. C, MTT cell viability assay comparing the response of wild type MEFs to lovastatin treatment alone or in combination with 30μM calpeptin.

A



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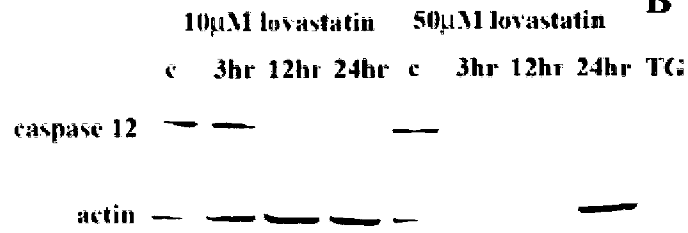

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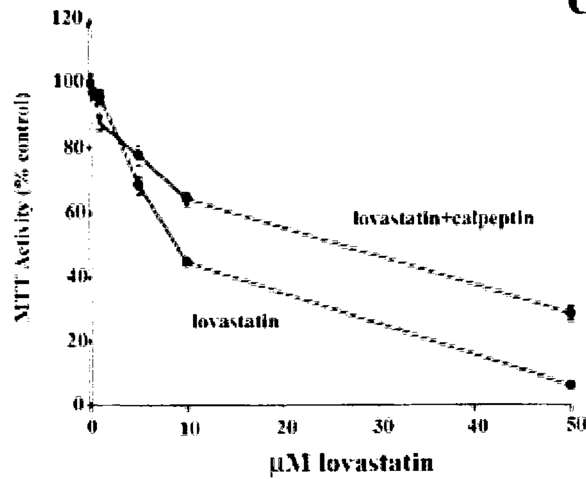
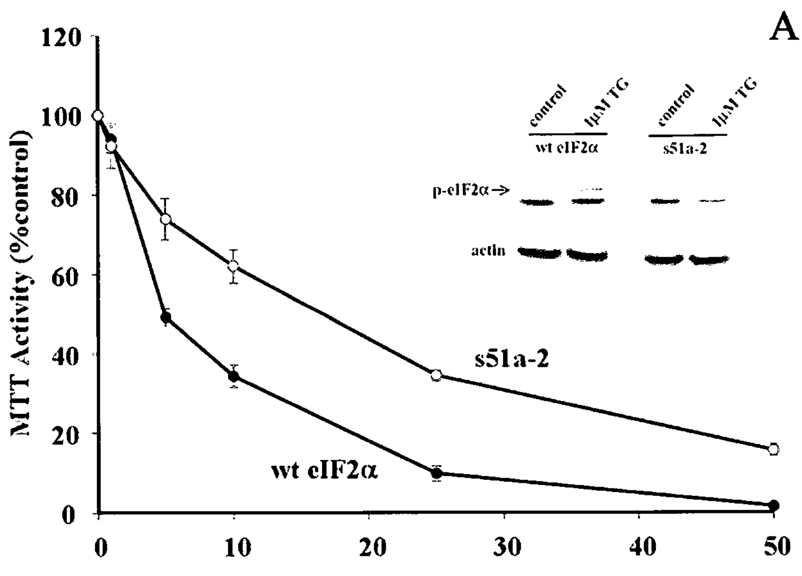
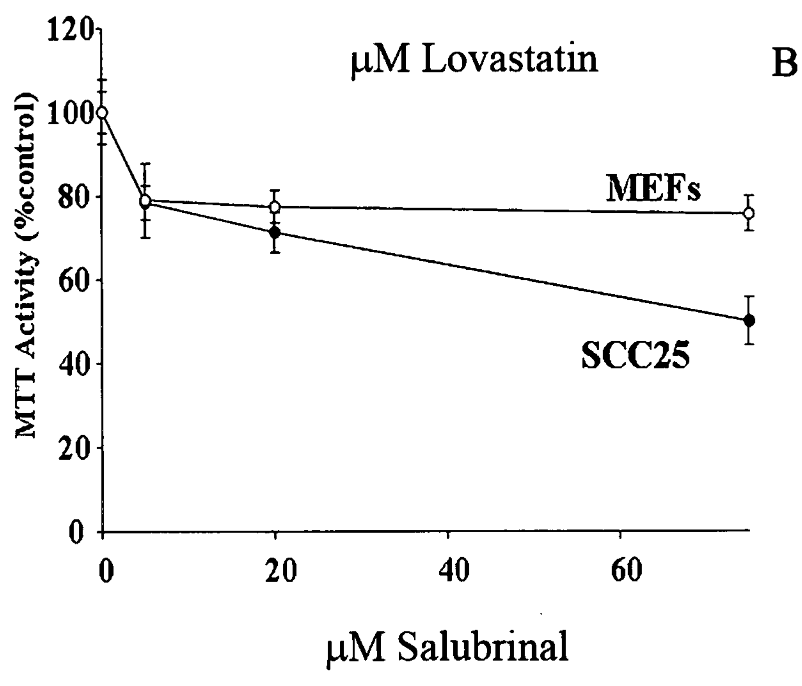


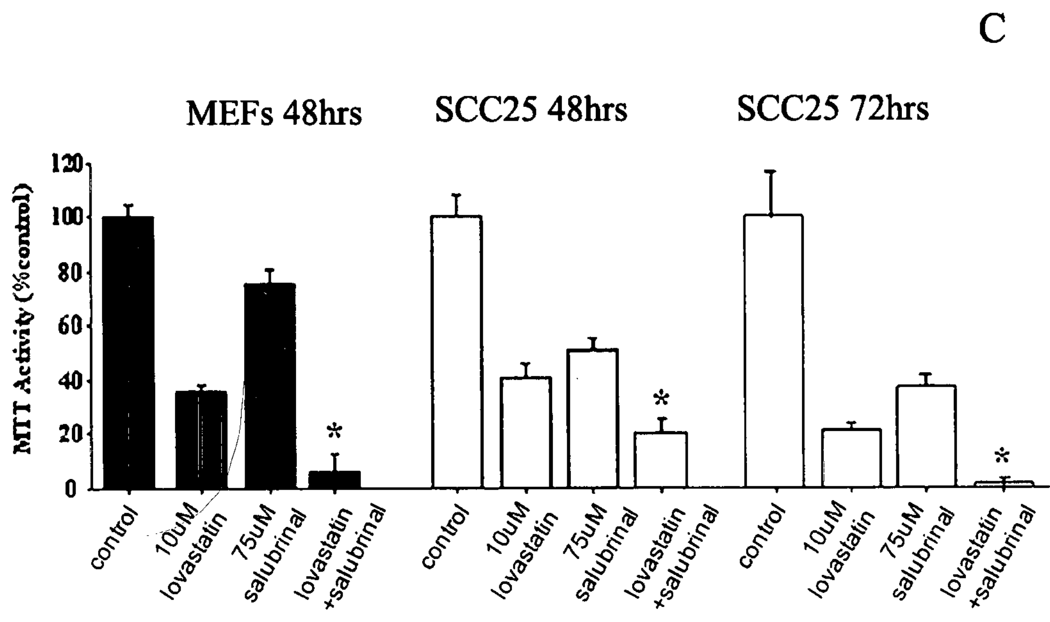
Salubrinal enhances lovastatin induced cytotoxicity

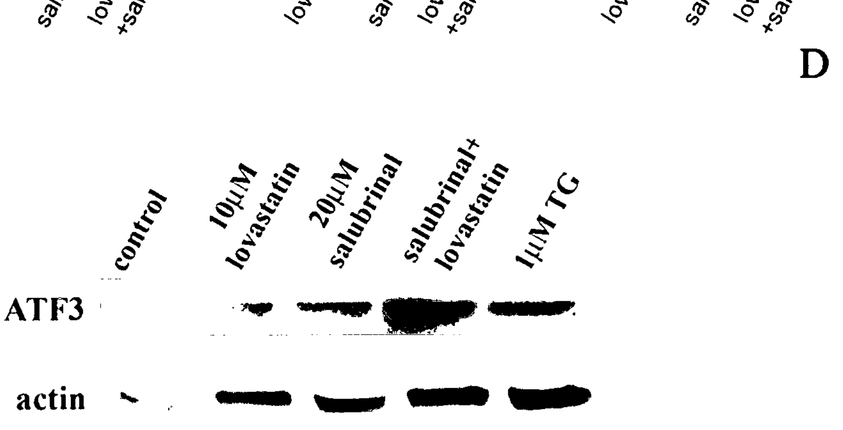
During stress induction, feedback regulators are induced to limit the inhibitory activity of eIF2α on protein translation as a means of reinstating translational machinery within the cell. GADD34 is a target of the ISR, particularly of CHOP, that is up-regulated and plays a role in the de-phosphorylation of eIF2α in a complex with type I Ser/Thr specific protein phosphatase PP1a (34,35). As expected, GADD34 is also induced by lovastatin in SCC25 cells (Supplemental Figure 3). To assess the role of eIF2α in regulating lovastatin-induced cytotoxicity, we evaluated the effect of this agent on MEFs exogenously expressing either a wild type eIF2α or a non-phosphorylatable allele where the serine at position 51 is mutated to an alanine (s51a). MEFs expressing the s51a non-phosphorylatable allele of eIF2α were less susceptible to lovastatin-induced cytotoxicity compared to cells expressing the wild type allele of eIF2α (Figure 7A). Salubrinal, a low molecular weight compound, has been demonstrated to inhibit the PP1a/GADD34 complex and to prolong the activity of p-eIF2α (34). In this study, we evaluated the potential of salubrinal to affect the cytotoxic effects of lovastatin in MEFs and SCC25 cells. As a single agent, treatment of these cell lines with salubrinal showed limited effects on cell viability (Figure 7B). However, combining salubrinal with lovastatin demonstrated enhanced cytotoxicity. Cytotoxicity was more pronounced than with either agent alone (Figure 7C). Expression of ATF3 was also significantly enhanced when 10μM lovastatin and 20μM salubrinal were combined for 24hrs and the response was greater than with the potent ISR inducer TG (Figure 7D). Salubrinal treatment enhanced the effects of lovastatin on ISR activation and cytotoxicity.

Figure 7. Salubrinal potentiates lovastatin induced cell death. A, MTT cell viability assays comparing the responses of wild-type eIF2-α and s51a mutant MEFs treated with 0–50 μM lovastatin for 48hrs. Cell viability was assayed with the activity of untreated cells taken to be 100%. Immunoblot analysis of p-eIF2α protein levels following 1μM TG treatment was used for selection of stably transfected clones expressing either *wt-eIF2α* or *S51A* mutant allele. Cell lysates from 1μM TG for 6hr as well as solvent control were analyzed. Total eIF2α was used as loading control. B, MTT cell viability assays showing toxicity of 0-75μM salubrinal in MEFs and SCC25 cells treated for 48hrs. C, MTT cell viability assays comparing the responses of MEFs and SCC25 cells treated with 10μM lovastatin and 75μM salubrinal alone or in combination for 48hrs. *Combination of treatments that displayed significant differences in MTT activity compared to either agent alone ($P<0.0001$, 2 way ANOVA). D, Immunoblot analysis of ATF3 protein levels following lovastatin and salubrinal treatments of MEFs. Cell lysates from 10μM lovastatin, 20μM salubrinal or combinations of these two treatments for 24hhrs as well as 1μM TG for 24hrs and solvent control were analyzed. Expression levels of actin were assayed as the loading control.


A
MTT Activity (%control)
120
100
80
60
40
20
0
0
10
20
30
40
50
μM Lovastatin
s51a-2
wt eIF2α
control
1μM TG
wt eIF2α
s51a-2
p-eIF2α→
actin


B
MTT Activity (%control)
120
100
80
60
40
20
0
0
20
40
60
μM Salubrinal
MEFs
SCC25


C
MEFs 48hrs
SCC25 48hrs
SCC25 72hrs
MTT Activity (%control)
120
100
80
60
40
20
0
control
10uM lovastatin
75uM salubrinal
lovastatin +salubrinal
*


D
control
10μM lovastatin
20μM salubrinal
salubrinal+ lovastatin
1μM TG
ATF3
actin

DISCUSSION

This study is the first demonstration that lovastatin differentially induces a potent ISR response in apoptosis sensitive SCC cell lines in comparison to resistant breast and prostate cancer cells. In sensitive SCC cells, ATF4 as well as its transcriptional targets ATF3 and CHOP were up-regulated both at the mRNA and protein levels. Inhibition of ATF3 expression attenuated lovastatin-induced apoptosis in HeLa cells and MEFs clearly indicating a role for the ISR in this response. CHOP is a transcription factor that can regulate among its many targets the expression of various BCL-2 family members that regulate mitochondrial function and intracellular calcium levels. In a previous study, we demonstrated the ability of lovastatin treatment to inhibit the expression of BCL-2 (36). In this study, SCC25 cells showed increases in intracellular calcium concentration, hallmarks of an apoptotic response (31, 37), coincident with lovastatin treatment. In MEFs, caspase 12 cleavage that is regulated in part by the calcium dependent proteolytic enzymes calpains was observed. Calpeptin, an inhibitor of calpains (38), also inhibited lovastatin-induced cytotoxicity in MEFs. Of therapeutic relevance, salubrinal that inhibits eIF2α dephosphorylation, enhanced lovastatin induction of ATF3 and its cytotoxicity in SCC cells and MEFs.

A variety of cell stressors result in the phosphorylation of eIF2α, which causes global translation attenuation. Phosphorylation of eIF2α on serine 51, a modification that prevents the assembly of the pre-initiation complex, halts global protein translation to aid in the cellular response to these stresses (17). Under these conditions, however, the translation of ATF4 is enhanced due to delayed translational re-initiation at upstream reading frames located at the 5'-end of the *ATF4* mRNA (18). Depending on the strength of the stress stimulus, these mediators

can either alleviate the stress or alternatively induce apoptosis (31). Enhanced expression of the transcription factors ATF3 and CHOP, ATF4 target genes, can mediate this apoptotic response (30, 39). Our studies clearly demonstrate a role for the ISR in regulating lovastatin-induced apoptosis as the ISR is preferentially induced in sensitive SCC cells and targeting the ISR attenuates its apoptotic response. A definitive role for GCN2 and its downstream effectors ATF4, ATF3 and CHOP in regulating the apoptotic effects of lovastatin have been demonstrated in our studies.

Co-administration of mevalonate can inhibit induction of ATF3 and apoptosis in lovastatin treated SCC25 cells (12, 14). This suggests that ATF3 induction by lovastatin is a consequence of the modulation of the mevalonate-pathway rather than activation of a secondary pathway or any side-effect of drug uptake by the cell. Mevalonate metabolites influence a variety of cellular processes and their depletion by lovastatin treatment may be the trigger for cellular stress activation and apoptosis, mediated by GCN2. Therefore identification of the cellular targets of mevalonate metabolites which regulate lovastatin induced apoptosis will have a profound clinical significance as it may uncover more specific therapeutic targets.

Impairment in de-phosphorylation of eIF2-α can attenuate inhibition of CHOP expression by GADD34 (40). Furthermore, the observation that S51A transfected MEFs are partially resistant to lovastatin induced apoptosis, suggests that phosphorylation of eIF2-α has an important role in lovastatin induced apoptosis. This is also is consistent with the protection of CHOP null MEFs against lovastatin induced apoptosis (14) since GADD34 is a target of CHOP (41).

As with most anti-cancer agents the clinical utility of statins is likely through their use in combination based modalities. In our studies we have undertaken both an empirical and a more rational approach where we identified the ISR as a regulator of lovastatin-induced apoptosis to identify combination treatment strategies. To this end, we demonstrated that in SCC, lung and colon carcinomas, combining statins with epidermal growth factor tyrosine kinase inhibitors induced synergistic cytotoxicity (42, 43). This work has led to a Phase I/II clinical trial evaluating this novel therapeutic approach at our institute (44). Identification of the ISR as a mediator of the apoptotic effects of lovastatin also has clinical implications. The ISR activator salubrinal can potentiate the cytotoxic effects of lovastatin. These results are similar to recent reports that proteasome inhibitors, also inducers of the ISR, cytotoxic effects in multiple myeloma cells were also enhanced by salubrinal (45, 46). Thus, combining ISR inducers with agents like salubrinal that target eIF2α function may represent a novel combinational therapeutic approach.

Experimental Procedures

Tissue Culture- Human tumour derived cell lines MCF-7 (breast adenocarcinoma), PC3 (prostate carcinoma), SCC25 and HeLa (cervical carcinoma) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The MEFs ATF3-/-, wild type ATF3+/+ (kindly provided by Dr. T. Hai, Ohio State University, Columbus, OH), GCN2-/-, +/+ (kindly provided by Dr. J. Bell, Ottawa Hospital Research Institute, Ottawa, Canada) and ATF4-/-, +/- (kindly provided by Dr. D. Park, University of Ottawa, Ottawa, Canada) as well as the tumour derived cell lines were maintained in HyQ DMEM/High Glucose (HyClone, Logan, Utah) supplemented with 10% fetal bovine serum (Medicorp, Montreal, QC). The cell lines used in this study were

exposed to solvent control, lovastatin (provided by Apotex, Mississauga, ON; diluted from a 10mmol/L stock in ethanol), thapsigargin (TG) (Calbiochem, San Diego, CA; reconstituted to a 1mmol/L stock in DMSO), salubrinal (ChemBridge, San Diego, CA), MG132 (Sigma, reconstituted to a 1mmol/L stock in DMSO) or Calpeptin (Calbiochem).

Design and expression of small hairpin RNAs- The two 19mer sequences targeting *ATF3* mRNA are; #1-5'-GCCAAAGAATATTCCATTT-3' and #2- 5'-GGGAGGGCCTGCAGTGATT-3' to pSuper vector from Oligoengine small hairpin RNA (shRNA) (#1: nucleotides 1524-1542; GenBank accession number NM_001030287. #2: nucleotides 1270-1289; GenBank accession number NM_001030287) target sequence. As controls, we used the *GFP*-targeted oligonucleotide 5'CATGCGTCCACTCTTCCTC-3' with accession number NC_011521. These sequences were BLAST confirmed for specificity. The forward and reverse synthetic 60 nt oligonucleotides (Integrated DNA Technologies, Coralville, IA) were designed, annealed, and inserted into the *Bgl*II/*Hind*III sites of pSUPER.retro.puro vector, following the manufacturer's instructions (Oligoengine, Seattle, WA). These constructs express a 19mer targeting two independent location within *ATF3* mRNA or *GFP* (control shRNA) mRNAs.

Generation of HeLa shRNAs expressing cells- Retroviral packaging cell line, RetroPack PT67 (Clonetech Labarotories, Mountain View, CA) was used for stable virus production according to the manufacturer's instructions. Briefly, packaging cells were transfected with *ATF3*-shRNA plasmids#1, #2 or *GFP*-shRNA, using FuGENE® HD Transfection Reagent (Roche, Missassauga, ON). After generation of stable clones and determination of viral titer, HeLa cells were infected with viral supernatant using 4 μg/ml polybrene. Stable transfected cells expressing shRNAs were selected using 3μg/ml puromycin.

Generation of stable eIF2α or S51A expressing MEFs- Both the *eIF2α* and *s51a eIF2α* containing plasmids (kindly provided by Dr. D. Ron, Skirball Institute, New York, NY) were digested with SmaI/BstBI to remove the CD2 region which was then replaced by the SmaI/BstB1 Neo/G418 cassette from PCDNA3.1. Proper sub cloning was confirmed by sequencing. Stable clones were selected using 800μg/ml neomycin/G418 then assayed for p-eIF2α expression following TG treatment.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay (MTT Assay)- In a 96-well, flat-bottomed plate (Fisher, Mississauga, ON), ~7500 cells/ 150μl of cell suspension were used to seed each well. The cells were incubated overnight to allow for cell attachment and recovery. Following treatment with lovastatin, 42μL of a 5mg/ml solution in PBS of the MTT substrate (Sigma) was added and incubated for up 2hrs at 37°C. The resulting blue-brown formazan precipitate formed was solubilized by the addition of 84μL of a 0.01M HCl/10%SDS (Sigma) solution and incubated for 8 hr at 37°C. The plates were then analyzed on a Dynex Technologies MRX Microplate Reader at 570nm using the Revel software (Dynex Technologies, Chantilly, VA) to determine the absorbance of the samples. Treatments were performed in replicates of six and the means expressed as the percent viability relative to the untreated control (100% viable).

Propidium Iodide Flow Cytometry- In 10-cm plates (Fisher), ~3.5×10^5 cells were used to seed each plate. The plates were incubated overnight to allow for cell attachment and recovery. The cell lines were treated with solvent control or lovastatin for 48hrs. After treatment, the media, PBS wash and trypsinized cells were collected in the same 50mL conical tube. The collected cells were fixed with 80% ethanol and incubated at -20°C for a minimum of 24hrs. The cells

were washed once then resuspended in staining buffer containing 50µg/ml propidium iodide (Sigma) and 100µg/ml RNaseA (Invitrogen, Carlsbad, CA). Ten thousand cells were evaluated using the Beckman Coulter Epics XL Flow Cytometer and the percentage of cells in pre-G_1 phase was determined using the ModFit LT program (Verity Software House, Topsham, ME).

Real Time RT-PCR- mRNA expression was measured using quantitative RT-PCR. cDNA was prepared from total RNA extracted from cells using Superscript II reverse transcriptase and p(dT) primer according to the manufacturer's protocol (Invitrogen, San Diego, CA). Transcripts were amplified in 20µl reactions containing 1X PCR buffer (20mM Tris-HCl, pH 8.4, 50mM KCl), 100uM dNTPs, 200uM gene specific primers, 100ng cDNA template, 0.02U/ul Platinum *Taq* DNA polymerase (Invitrogen) and Sybr green I (Molecular Probes, Carlsbad, CA). Amplification was carried out on a LightCycler thermocyler (Roche) using 50 cycles of 95°C for 20s, 58°C for 10s, 72°C for 24s. Fold changes, expressed as means (+/- SEM, n=3), were calculated for drug treated groups versus vehicle control using ΔΔCt values following normalization to *GAPDH* transcript levels. The primers for gene-specific RT-PCR analysis were as follows: *ATF3*:TAGGCTGGAAGAGCCAAAG(5') and TTCTCACAGCTGCAAACACC(3'); *ATF4*: AGTGATATCCACTTCACTGCCCAG(5') and AAGGAGTTCGCCTTGGATGCCC TG(3'); *CHOP*: CTCTGACTGGAATCTGGAGAGTG(5') and CTGAGTCATTGC CTTTCTCTTCG(3'); *GAPDH* TTGATGTCATCATACTTGGCAGGT(5') and CAG TCAAGGCTGAGAATGGGA(3').

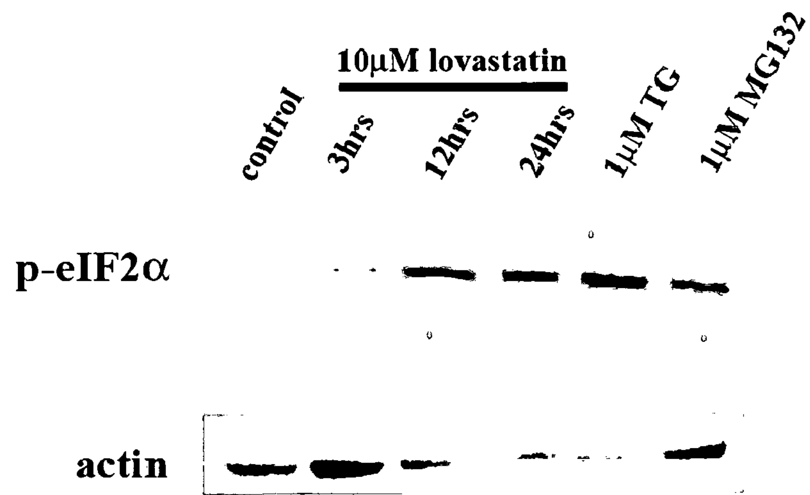
Determination of Intracellular Calcium Levels- Treated SCC25 cells were trypsinized and counted with a Vi-Cell XR Viable Cell Analyzer (Beckman Coulter, Toronto, ON). For each sample, 1×10^6 viable cells were transferred to a 15mL Falcon tube, pelleted by centrifugation, then resuspended in 200μL Cell Loading Medium (CLM; DMEM supplemented with 2% FBS and 25mM HEPES pH 7.4) containing 2μM Oregon Green 488 BAPTA-AM (Molecular Probes) then incubated for 1hr at 37°C followed by dilution to 1mL with CLM prior to FACS analysis. For the thapsigargin positive control, untreated SCC25 cells were loaded with Oregon Green as above, then 1μM TG was added to the diluted cells approximately 15min prior to analysis. Samples were analyzed for fluorescence at 525nm using an Epics XL Flow Cytometer (Beckman Coulter, Mississauga, ON).

Western Blot Analysis- Total cellular protein was extracted using a buffer that consisted of 50mM Tris-HCl pH 7.5, 150mM NaCl, 0.25% sodium deoxycholate (Sigma), 1% IgePal, 0.1% SDS (Sigma), 1mM EDTA, 5mM sodium fluoride (Sigma), 1mM sodium orthovanadate (Sigma), and protease inhibitor cocktail (Sigma; diluted from a 10x stock). Approximately 100μL of extraction buffer was used to per 10cm plate. Total protein was quantified with the BCA Protein Assay Reagents (Pierce, Nepean, ON) using bovine serum albumin (Sigma) for the standard. Protein extracts representing 50 to 100μg total protein were separated on SDS-PAGE gel using the BioRad Mini Protean 3 System (Bio-Rad Laboratories, Hercules, CA) and electro-blotted onto Hybond P PVDF membranes (Amersham, Piscataway, NJ). Membranes were blocked in 5% skim milk powder in PBS/0.02% Tween (Sigma) for an hour at room temperature. Primary antibody, diluted in 5% skim milk powder in PBST, was incubated with the membrane overnight at 4°C. The antibodies used were specific for ATF3, CHOP and caspase 12 (Santa

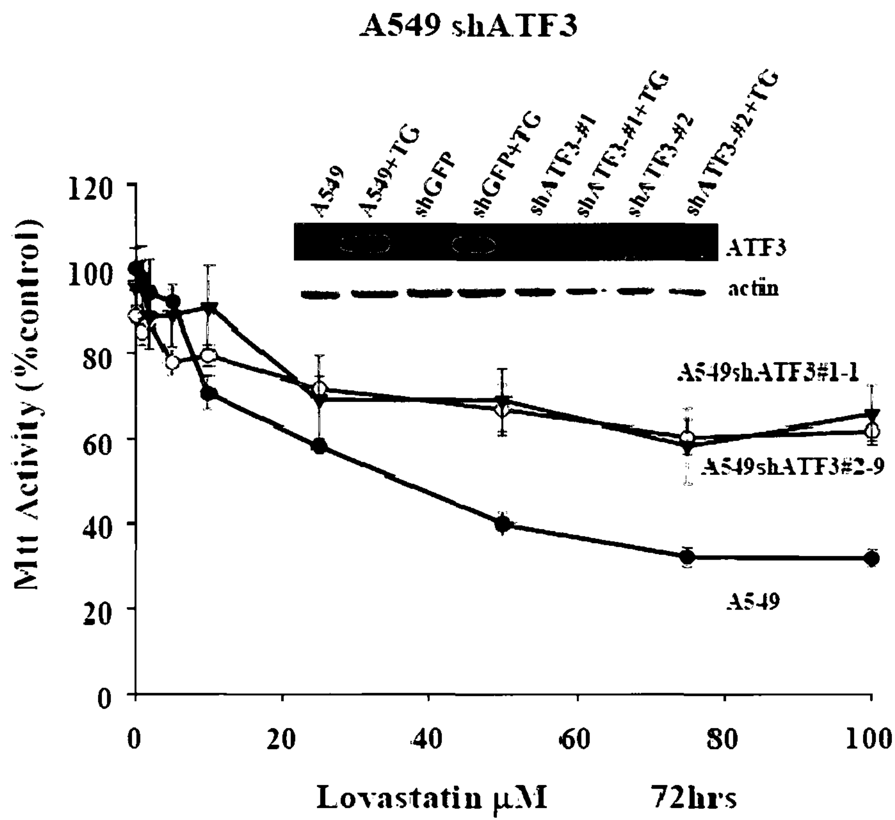
Cruz Biotechnologies, Santa Cruz, CA) at dilutions of 1:500. The PARP (Cell Signaling Technology, Danvers, MA) and actin (Sigma) antibodies were used at a dilution of 1:1000. The peroxidase-conjugated AffiniPure Goat Anti-mouse/rabbit IgG (Jackson ImmunoResearch, West Grove, PA) secondary antibodies were applied at a 1:5000 dilution in 5% skim milk powder in PBST and incubated for a minimum of 1hr at room temperature. The blots were then processed for detection with the Supersignal West Pico Chemiluminescent Substrate (Pierce), using the Gene GNOME Imager and Genesnap Imaging Software (Syngene, Frederick, MD). After the desired exposure was obtained, the membrane was stained with Ponceau Red (Fisher) to ensure equal loading of the samples.

Immunofluorescence- In a 6-well flat-bottomed plate (Fisher), glass cover slips (Fisher) were placed into each well and ~250000 cells were used to seed each well. The cells were incubated overnight to allow for cell attachment and recovery. Following a 24hr treatment of solvent control or lovastatin, the cells were subsequently washed with PBS then fixed with 4% paraformaldehyde (Sigma) buffered in PBS for 15 minutes at 37°C and stored in PBS at 4°C. Prior to immunofluorescence staining, the cells were permeabilized with PBS+0.2%Triton X-100 (Sigma) for 15 minutes. The cells were blocked for 30 minutes with PBS+3%FBS then incubated with the CHOP or cytochrome c antibodies (Pharmingen, San Diego, CA) at a dilution of 1:50 in PBS+3%FBS for an hour. The cells were then blocked with PBS+5%FBS for 30 minutes. Following the second blocking, the cells were incubated with Alexa Fluor CY3 goat anti-mouse IgG (Molecular Probes, Carlsbad, CA) at a working dilution of 10μg/ml in the dark for an hour. The cells were then mounted to a microslide with DAPI mounting media (Vector Laboratories, Burlingame, CA) and analyzed under fluorescent microscopy using the Axiovision software (Allied High Tech Products, Rancho Dominguez, CA).

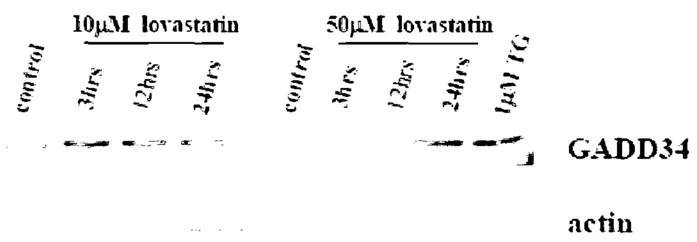
Supplemental Figure 1. Immunoblot analysis of p-eIF2α in MEFs following 3, 12 and 24hr 10μM lovastatin treatment, 1μM TG and 1μM MG132. Expression levels of actin were assayed as the loading control.


10μM lovastatin
control
3hrs
12hrs
24hrs
1μM TG
1μM MG132
p-eIF2α
actin

Supplemental Figure 2. MTT cell viability assays comparing the responses of A549 cells stably cloned with short hairpin RNA expressing two different anti-sense sequences against *ATF3* as well as *GFP* as control. Cells were treated with 0–50 μM lovastatin for 48 hr. Immunoblot analysis was used to assess the efficiency of sh-*ATF3* clones for knockdown of ATF3 expression in cells, were treated with 1μM TG for 24hrs, or solvent only as control. Expression levels of actin were assayed as the loading control


A549 shATF3
A549
A549+TG
shGFP
shGFP+TG
shATF3-#1
shATF3-#1+TG
shATF3-#2
shATF3-#2+TG
ATF3
actin
A549shATF3#1-1
A549shATF3#2-9
A549
Mtt Activity (%control)
Lovastatin µM 72hrs
120
100
80
60
40
20
0
0
20
40
60
80
100

Supplemental Figure 3. Immunoblot analysis of GADD34 expression in SCC25 cells following 3, 12 and 24hr 10μM or 50μM lovastatin treatments. Treatment with 1μM TG was employed as a control for ISR induction. Expression levels of actin were assayed as the loading control.


10μM lovastatin
50μM lovastatin
control
3hrs
12hrs
24hrs
control
3hrs
12hrs
24hrs
1μM TG
GADD34
actin

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FOOTNOTES

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The abbreviations used are: HMG-CoA, 3-hydroxy-3-methyl glutaryl coenzyme A; ISR, integrated stress response; SCC, squamous cell carcinoma; ATF, activation of transcription factor; MEFs, murine embryonic fibroblasts; ER, endoplasmic reticulum; eIF, eukaryotic initiation factor; TG, thapsigargin; GGPP, geranylgeranyl pyrophosphate; FPP, farnesyl pyrophosphate

CHAPTER 4

Discussion

The hallmarks of cancer cells include unchecked proliferation and evasion of apoptosis, leading to the formation of tumours and eventually metastasis once these cells invade the circulatory system, while conversely, normal cells grow and divide to a point then exit the cell cycle by undergoing differentiation or apoptosis. Due to unlimited growth potential and its corresponding requirements for higher metabolism, cancer cells can be more vulnerable to further increases in stress conditions. Thus interventions that can induce cellular stress responses and force the cell to undergo apoptosis, containing tumour growth or eliminate cancer cells, represent feasible therapeutic approaches.

4.1 Integrated stress response and lovastatin

In this study we have demonstrated that lovastatin induced apoptosis in HNSCC SCC25 cells, as well as in HeLa and MEF cell lines is mediated through activation of the ISR in a PERK-independent and GCN2-dependent fashion. In this project, GCN2 was identified as the primary kinase required for phosphorylation of eIF2-α, independent of PERK and that activation of the ISR by lovastatin treatment in sensitive tumour cell lines occurs without activation of conventional ER stress (Chapter 2). There is a recent report linking statins and induction of UPR markers, BiP, XBP-1(at protein levels only), and p-eIF2-α in mouse macrophage cell line RAW264.7 and curiously CHOP was not induced however, statins in this study did not induce apoptosis and in fact protected the cells from hypoxic cell death probably due to cell type specific traits [245]. The signaling mechanism responsible for the engagement of GCN2 by lovastatin it is still unclear, however, one possibility is that cancer cells lose their ability to

uptake amino acids following lovastatin treatment, due to attenuated expression, inactivation of cell surface receptors or modulation of amino acid permeases thereby leading to activation of GCN2 . Modulation of general amino acid transporters by TOR signaling [246, 247] and targeting of mTOR by statins [248] may provide an indirect link to support this hypothesis. Interestingly, one study suggests that, specifically, blockade of leucine amino acid transporter (LAT1) which is expressed in Hep-2-a HNSCC-cell line enhances cisplatin (also an ISR inducer) induced cytotoxicity [249]. Thus it is plausible that lovastatin, by targeting intracellular trafficking, may limit the levels of accessible free amino acids from recycled proteins and/or inhibit their uptake, thereby promoting activation of GCN2.

In addition to the activation of GCN2 by reduced cellular levels of charged tRNAs, direct modulation of the binding affinity of GCN2 to uncharged tRNA can also activate kinase activities. In this case, despite the cell having "normal" levels of uncharged tRNA, increases to the binding affinity of uncharged tRNAs for GCN2 effectively produces a similar condition as amino acid starvation thereby increasing GCN2 kinase activity [86]. For instance, rapamycin modulates phosphorylation levels of GCN2 in yeast, leading to higher binding affinity for uncharged tRNA [250]. Certain modifications to tRNA are associated with tumourigeneis in lung and ovarian cancers [251, 252] . This may be a reason for the lower threshold of GCN2 activation following lovastatin treatment, such that minor disturbances in readily deregulated tRNA levels, can produce cancer cells with a shortage of charged tRNAs leading to activation of GCN2. At this point the possibility that lovastatin may induce conformational changes to GCN2 directly (e.g phosphorylation) to activate this kinase cannot be disregarded and can be examined in future studies.

Another regulatory mechanism which controls the activation of GCN2 is the MEK/ERK signaling pathway as its inhibition attenuates phosphorylation of eIF2-α in amino acid deprived HepG2 cells [253]. However, it seems unlikely that GCN2 is activated through MEK/ERK signaling following lovastatin treatment, or for this pathway to be a key factor in phosphorylation of eIF2-α, since this signaling pathway is not significantly affect by lovastatin treatment in HNSCC cells [23].

4.2. Mevalonate pathway and ISR activation

There is emerging evidence linking ISR to perturbations of cellular sterols. TGD31/45, a small molecule inhibitor blocking lanosterol demethylation, a late step in cholesterol biosynthesis, upstream of the mevalonate pathway, can induce ISR [254]. By inhibition of a more upstream target, HMG-CoA reductase, this study shows that lovastatin induces ISR through a mechanism that is reversible by supplementation with the downstream mevalonate pathway product, GGPP (Chapter 2). Intriguingly, free fatty acid (FFA) accumulation in pancreatic beta cells can also induce ISR, and synergistic targeting of p-eIF2-α/ATF4 by means of salubrinal increases FFA induced apoptosis in these cells [255]. In view of previous studies linking inhibition of the mevalonate pathway by statins to the enhancing of fatty acid and triglyceride synthesis [256, 257], it seems reasonable to speculate that activation of the ISR by statins involves increases in the fatty acid content of sensitive tumour cells and this likely involves GCN2 kinase activation. Consistent with this, a recent study in GCN2 null mice identified a novel function of GCN2 in the regulation of fatty-acid homeostasis in the liver during leucine deprivation by regulating an isoform of SREBP, SREBP1c, and fatty acid synthase (FAS) [235], suggesting further links between GCN2 kinase activity and regulation of sterols, although, this requires further studies.

4.3 Translational regulation by statins

Upregulation of specific target mRNAs occurs even under conditions of mild induction of eIF2- α phosphorylation, for example, studies have shown that *gcn4*, in yeast, and *ATF4* in mammalian systems are up-regulated following moderate increases in p-eIF2-α levels [86, 258] . In this study, although the level of phosphorylation of eIF2- α by lovastatin is less than that produced by TG, strong induction of ATF4 is still observed (Chapter 2). However, the resulting severe inhibition of translation by lovastatin suggests that a secondary mechanism is likely to be involved. The AKT/mTOR/4EBP1 pathway, which also regulates translation initiation [124] , is a likely candidate as both lovastatin and fluvastatin can target the mTOR pathway by inhibiting phosphorylation of 4E-BP1 which enhances its binding to and translational repression of the mRNA cap-binding protein, eukaryotic initiation factor 4E (eIF4E) and specifically for fluvastatin this results in inhibition of cap-dependent translation in renal cell carcinoma and MEFs *in vitro*[248]. Lovastatin affects the phosphorylation status of 4E-BP1 in MEFs (Appendix II). Enhanced expression of eIF4E has been associated with increased progression of several cancers, including HNSCC and prostate tumours [259, 260] and coincidently, eIF4E can also modulate signaling of Ras, a potent regulator of the Rho family of small GTPases, which require isoprenylation for their function [261].

Inhibition of AKT signaling by lovastatin has been demonstrated in HNSCCs (Appendix I). Therefore lovastatin treatment may result in repression of eIF4E, by 4E-BP1, and this could provide an additional pathway for translation inhibition supplementary to induction of p-eIF2-α by this drug. A recent report suggests that ER-stress related cell death in mouse pancreatic β-cells, in part is regulated through induction of 4E-BP1 in an ATF4-dependent fashion suggesting a crosstalk between ISR and mTOR signaling pathway [157]. The mTOR pathway also regulates

upstream binding factor (UBF) and this transcription factor regulates the rate of rRNA synthesis and thus protein synthesis [262]. Interestingly, microarray data from our lab, in lovastatin sensitive, SCC25 and SCC9 cell lines shows that *UBF* is down-regulated following lovastatin treatment, while it remains unchanged in insensitive MCF-7 cells, further demonstrating a translational regulatory mode of action for lovastatin (Dimitroulakos, unpublished).

Lovastatin targeting of both ISR and AKT signaling pathways can also explain findings in our lab showing that the combinations of EGFR-targeted therapy with lovastatin produce synergistic cytotoxicity in HNSCC cells (Appendix I). Various cancers show hyper-activated AKT due to mutations in the AKT regulator PTEN however, AKT requires EGFR signaling for its activation [263]. The use of EGFR inhibitors like gefinitib to control cancers, has become limited due to the prevalence of EGFR mutations in certain cancer cells therefore combination therapies are used as means of overcoming this limitation [264]. Combination of lovastatin and gefinitib represents an attractive possibility. By inhibition of AKT activity and indirectly targeting mTOR pathway, cap-dependent translation can be repressed and concomitantly lovastatin, by inducing ISR, would further contribute to this co-operative effect and promote apoptosis by simultaneous down-regulation of survival signals (AKT) and inducing pro-apoptotic transcription factors. This is supported by reports showing simultaneous targeting of AKT and ISR in EGFR inhibitor-resistant NSCLC cells (for example combination of ER-stress inducer, tunicamycin and EGFR inhibitor, erlotinib) can overcome resistance to epidermal growth factor receptor inhibitors [265, 266]. This identification of synergistic pathway activations by various drug combinations is an important step in the development of novel refined “multiple-hit” strategies, tailored for specific cancer cells.

4.4 Induction of ISR by lovastatin leads to cell cycle arrest

Phosphorylation of eIF2-α by GCN2 causes a decrease in the synthesis of cyclin D1, leading to G1 cell cycle arrest [131]. In line with this observation we observe that lovastatin caused G1 arrest in cancer cells (Chapter 3). Consistent with the evolutionary role of the ISR, G1 arrest during lovastatin treatment may initially occur to allow the cell to restore their physiological homeostasis however, due to extensive stress activation and through expression and accumulation of CHOP, apoptotic pathways become engaged. This is in agreement with the concept that prolonged activation of ISR, that does not restore cellular homeostasis, leads to apoptosis. In addition to being critical for translation of capped mRNAs, initiation factor eIF4E is also a positive regulator of cyclin D1 [267] and an indirect target of the UPR as it has recently been identified as a target of ATF4, mediated through 4E-BP1 [157] . This provides additional insight into the mechanisms of lovastatin induced apoptosis as translation inhibition induced by ISR can be linked to cell cycle progression through a combined pathway involving ATF4/4E-BP/eIF4E/cylin D1. ATF3 also can bind to the promoters of cyclin D1 and the CDK inhibitor p21, causing suppression and induction of these two cell cycle regulators respectively promoting G1 cell cycle arrest [205, 268]. As ATF3 is also induced by lovastatin (Chapter 3) this is an additional mechanism to explain how the induction of ISR leads to cell cycle arrest in lovastatin sensitive cell lines. Combinational therapies which targeting ISR and cyclin D1 (e.g lovastatin and UV radiation) could be used to exploit this linkage between stress activation and cell cycle arrest and therefore may be beneficial for the control of rapidly proliferating tumour cells.

The possibility that cell cycle arrest by lovastatin may be mediated through proteasome inhibitor activities of the pro-drug form of lovastatin was also examined (Chapter 2). While both lovastatin prodrug and open active forms and MG132 did induce ISR, induction of HSP70 and

BiP, cytoplasmic and ER chaperones whose expression is correlated with increases in misfolded proteins, was only seen moderately with the known proteasome inhibitor, MG132 (Chapter 2). In addition, in contrast to previous studies [12], lovastatin (pro-drug and active form) was shown not to have an inhibitory effect on proteasomal activity nor on the accumulation of poly-ubiquitinated proteins following treatment indicating that proteasome inhibition is not correlated with induction of ISR and cell cycle arrest in lovastatin treated SCC25 cells. However, it has been suggested that upon addition to cell culture media the pro-drug form is readily converted to the open-ring active form which might explain the discrepancy [269]. Furthermore, it has been suggested that the loss of cyclin D1 following phosphorylation of eIF2-α requires intact 26S proteasome function, which can be inhibited by addition of MG132 [270], and this may also explain loss of cyclin D1 following lovastatin treatment [271].

4.5 Lovastatin induced apoptosis is ATF3 dependent

ATF3 was upregulated following lovastatin treatment in HNSCC cell line SCC25, and plays a pro-apoptotic role in lovastatin induced cell death in MEFs, A549 and HeLa cells, but was not induced in insensitive MCF-7 breast carcinoma cells (Chapter 2 and 3). It has been shown that MCF-7 cells lack Caspase 3 expression due to mutation, however, the lack of induction of the ISR by lovastatin in these cells indicates that lovastatin insensitivity in MCF-7 cells is not primarily related to the absence of Caspase 3 and may indicate further disruptions upstream of Caspase 3 which block the activity of lovastatin [272]. Interestingly further increases in ATF3 induction by an auxiliary agent like salubrinal can potentiate lovastatin induced toxicity (Chapter 3). Induction of ATF3 by lovastatin, unlike UV irradiation, was shown to be dependent upon activation of ISR, and more specifically the GCN2-ATF4 pathway, in a cell-type dependent manner (Chapter 3 and [273]).

Activation of JNK and p38 MAPK by non-ER stress stimuli, such as UV irradiation and anisomycin, was found to be independent of PERK or ISR activation, however these signaling pathways can be activated in a PERK-dependent manner by TG [91]. Interestingly, it is reported that ATF3 induction by anisomycin and UV irradiation also requires activation of p38 and JNK signaling pathways respectively [197, 274]. While induction of ATF3 by TG requires PERK expression, it is not clear whether TG requires either of these signaling pathways for ATF3 induction [275]. Our data indicate that induction of ATF3 by both TG and lovastatin are contingent on the expression of ATF4 (Chapter 3). While activation of p38 by statins is reported in several cell lines [271] , like NF-κB, it is presumed a secondary consequence of the accumulation of intracellular ROS rather than direct targeting of this signaling pathway [52]. Furthermore, the p38 and JNK signaling pathways were not affected by lovastatin treatment in sensitive AML cell lines as well as in AML primary blasts [276]. As such, this suggests that p38 and/or JNK signaling pathways are not primary effectors in statin induced ISR and as this study indicates, ATF3 induction relies on upstream GCN2 rather than any secondary pathway (Chapter 3). However, further studies are required to identify any potential cross-talk between p38/JNK and GCN2-ATF3 signaling pathway in different cell types by statins.

Interestingly ATF3 is also identified as a target of TGF-β, a pleiotropic cytokine involved in a diverse array of cellular processes including embryonic development and tissue homeostasis [277]. TGF-β, with its dual function in cell survival and apoptosis activation, has also been implicated in tumourigenesis [278]. Currently there is no data to indicate the existence of a link between TGF-β and ISR activation however it is possible that simultaneous targeting of TGF-β and ISR, following careful evaluation of ATF3 status in target tumour cells, may result in a synergistic response to the activation of ATF3 , increasing its pro-apoptotic response. Another

ATF3 inducer, the tumour suppressor KLF6, directly binds to the promoter of *ATF3* resulting in upregulation and promotion of apoptosis. However, the *KLF6* gene in several human cancers, including prostate cancer, undergoes frequent mutations that inhibit binding to the *ATF3* promoter region and thus preventing induction of apoptosis by ATF3, even under hypoxic conditions where ATF3 is normally induced independent of ISR [273, 279] . In examples such as these, therapeutic agents such as lovastatin, by induction of ISR and ATF3, provides a means for a cancer cell, with mutation to key tumour suppressors such as KLF6, to undergo apoptosis, given that KLF6 expression is independent of ISR. However, due to the dual nature of ATF3 as either a promoter or repressor of apoptosis, knowledge of the status of ATF3 in the target tumour cell is critical to determine whether statin treatment will be effective.

Based on the observations of this study it is conceivable that lovastatin, similar to several other ISR inducers (e.g. MG132, UV and amino acid deprivation), is only engaging the AARE promoters of *CHOP* through induction of ATF3 and ATF4, although this remains to be determined. During amino acid deprivation the levels of CHOP are modestly activated compared to those induced by ER stress [159] . Since, like amino acid deprivation, lovastatin appears to primarily activate GCN2 and downstream targets to induce the expression of CHOP this can explain the more modest upregulation of CHOP expression (or other ISR components) compared to those observed following treatment with TG (Chapter 2).

Lovastatin induced apoptosis was attenuated in CHOP null MEFs (Chapter 2). It has been suggested that the protection gained by loss of CHOP expression indicates the role of CHOP has evolved to promote apoptosis under severe or prolonged stress conditions or alternatively continued induction of CHOP under stress conditions results in upregulation of GADD34, de-phosphorylation of eIF2-α and failure of cellular homeostasis at the level of protein synthesis

[86]. In this study disrupting the function of GADD34, a negative feedback regulator and target of CHOP, by means of salubrinal enhances lovastatin induced apoptosis, most likely due to the increased ATF3 expression and consequently, further upregulation of CHOP thereby mimicking a condition of prolonged stress. Furthermore, CHOP suppresses the expression of anti-apoptotic BCL-2, which opposes the induction of BCL-2 by AKT [280].Thus explaining the mechanism of BCL-2 repression by statins [271]. Additionally, death receptor 5 (*DR5*), is another target gene of CHOP, which is implicated in CHOP induced apoptosis in several human carcinomas during ISR and may also play a role in lovastatin-induced cell death and therefore warrants further examination [182] .

Mutations within any of the key players of the ISR results in the accumulation of reactive oxygen species within the cell [281, 282].These data suggest that the ISR has a key role in the maintenance of redox homeostasis. ER oxidoreductin (ERO1) is involved in setting the redox homeostasis of the ER, by generating hydrogen peroxide within the ER lumen [283] . Interestingly, an isoform of ERO1, *ERO1α* ,is a target gene of CHOP [171]. During the course of ISR, it is likely that CHOP attempts to increase the client protein synthesis of the ER by induction of *ERO1α*, which can assist in disulfide bond formation. However, if the ROS levels surpass the capacity of the anti-oxidant machinery within the cell, oxidative stress will result [86]. Thus, it is likely that consistent expression of CHOP following lovastatin treatment can impose a challenge to the maintenance of redox homeostasis of the cell by producing ROS. Although the generation of ROS species by statins has been observed to, in part, account for their cytotoxicity [52] , as yet there has been no report of ERO1α upregulation by statins. However, emerging *in vitro* and *in vivo* data indicate that NRF2 –a binding partner of ATF4-and its target gene *HO-1* modulate ROS levels following simvastatin treatment with both pro- and anti-

apoptotic roles reported [284-288]. This is also consistent with the observation that ATF4 null cells show an oxidized ER state during ER stress [162]. Interestingly, NRF2 is critical for the prosurvival function of PERK [289] probably by mediating the anti-oxidant function of p21 [290]. Since both ATF4 and p21 expression are modulated by lovastatin (Chapter 2,[271]) the initial cellular response to statins may represent an attempt to overcome the stress with enhanced expression of both ATF4 and p21 contributing to the regulation of NRF2 to promote an anti-oxidant response in response to generation of ROS, thereby promoting cellular survival. However, it is possible that in sensitive cancer cells, due to higher metabolism and elevated basal levels of oxidative stress, high levels of ATF4 result in the recruitment of other binding partners, like ATF3, in order to promote apoptosis through CHOP or other targets. Consistent with this proposed mechanism, two different proteasome inhibitors, bortezomib and Eer1, through binding of ATF3 and ATF4 can promote cell death via activation of the BH3-only protein NOXA in a p53-independet mechanism in human cancer cells [193]. CHOP null cells are partially resistant to lovastatin induced apoptosis, therefore it is plausible that ATF3-ATF4 binding following lovastatin treatment, in addition to modulating CHOP expression may induce activation of the BH3-only protein NOXA and further contribute to lovastatin induced cytotoxicity. However, to date there is no data indicating activation of NOXA by statin treatment and thus warrants further examination.

4.6 Lovastatin disrupts intracellular calcium regulation

In this project, release of intracellular calcium in SCC25 cells following lovastatin treatment was detected (Chapter 3), in agreement with previous reports showing a modulation of intracellular calcium by statins [47]. Both PERK and calpains are known to be activated by perturbations in calcium homeostasis within the ER. In our study, we showed the involvement of

calpains, but not PERK, in lovastatin induced cell death. It is possible that different calcium concentrations are required for activation of PERK and calpains, such that a modest increase only activates calpains. In fact, *in vitro* it has been shown that the two different, ubiquitously expressed, isoforms of the large subunit of calpain , m-calpains and μ-calpains require different calcium concentrations at mM and μM, respectively, in order to be activated [291] and Caspase-12 specifically is activated by m-calpains [238]. The source of calcium for activating calpains can be either intracellular (from ER) or extracellular. Interestingly, TRPM7 is a calcium sensing channel that is expressed in SCC25 cells, as well as in gastric cancer cells, and its blockade has anti-proliferative and apoptotic effects [292, 293]. Disruption of intracellular calcium homeostasis may in part explain the observed mitochondrial fragmentation induced by lovastatin (Chapter 3), a hallmark of apoptotic cells. A more direct link between mitochondrial mediated apoptosis and p- eIF2-α has been established such that translational repression of anti-apoptotic myeloid cell leukemia sequence 1, MCL-1, can lead to cell death mediated through mitochondrial apoptotic mechanisms [294] . Since Caspase 12, a target of calpains in murine systems is inactive in humans and calpeptin did not abrogate lovastain induced cell death in SCC cells (unpublished data), there is a need for further experimentation to dissect the role of calcium regulation in lovastatin associated toxicity.

4.7 Therapeutic applications and Future directions

A desired chemotherapeutic agent is one whose cytotoxicity is targeted specifically towards cancer cells with limited side effects to the surrounding healthy tissue. Phase I clinical trials employing lovastatin, or other statins, alone as chemotherapeutic agents for a variety of different cancers resulted in only modest clinical outcomes, in part due to drug limiting

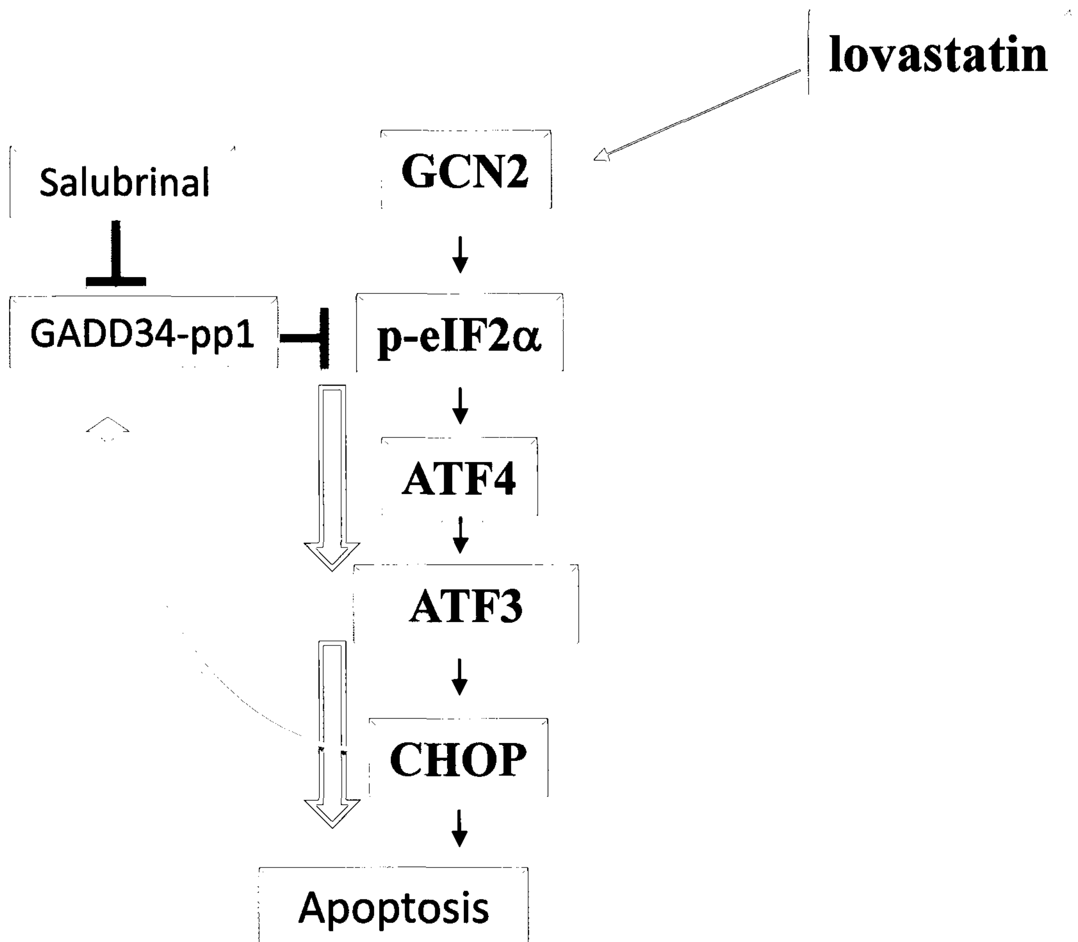
toxicities. Understanding the molecular mechanism by which lovastatin and similar agents target the translational machinery of cancer cells will help to facilitate development of more effective treatment strategies whereby lovastatin can be utilized at lower doses in combination with agents that potentiate the stress response. In this study we have shown that lovastatin induced apoptosis in sensitive cell lines involves the activation of ISR, through GCN2-dependent phosphorylation of eIF2-α, and disruption of cellular translation ultimately leading to cellular death (Fig 4.A). Activation of the ISR in tumours has previously been demonstrated to occur due to the hypoxic microenvironments that develop within a solid tumour [96]. While activation of the ISR has been shown to protect cells from damage due to oxidative stress there is also evidence where prolonged activation of this pathway can overwhelm any protection that may be imparted and induce apoptosis, for example through repression of anti-apoptotic BCL-2 or MCL-1 [162, 294]. Development of therapies involving lovastatin in combination with other agents that potentiate eIF2-α phosphorylation may be beneficial in targeting tumour cells that are already in a stress condition and push the ISR pathway from survival mode towards apoptosis, while adjacent normal cells can tolerate moderate levels of ISR activation. This study provided such an example, as the combination of lovastatin and salubrinal exhibited very promising results for potentiated apoptotic responses in both MEFs, as a model cell line, and HNSCC cell line, SCC25, indicating this combination should be considered in future therapeutic modalities. However, further *in vivo* work and examining the effects in a broader range of tumour cell lines is a necessity. An example of this approach has been used in the treatment of MM by bortezomib. While induction of the ISR by this agent, in part, accounted for its cytotoxicity [47], inhibition of dephosphorylation of eIF2-a, by salubrinal increased the cytotoxicity of this drug through promoting apoptosis [149].

4.8 Conclusions:

The complexity and heterogeneity of ISR responses by various inducers in different tumour cell lines requires careful examination of the role of ISR in a case dependent manner. This study indicates an important example and role for the ISR through GCN2-dependent activation in lovastatin induced apoptosis in sensitive cancer cells and shows direct links between induction of ISR and lovastatin associated toxicity. Furthermore, identifying ATF3 as a proapoptotic gene with the context of lovastatin induced ISR and enhancing its expression by combination with salubrinal appears to be very promising therapeutically.

Figure 4.A. Model of lovastatin induced cytotoxicity. Lovastatin, by targeting parallel signaling pathways including the ISR and mTOR pathways, causes translation inhibition followed by apoptosis in sensitive cell lines. Salubrinal potentiates this response by further upregulating ISR downstream effectors ATF3 and CHOP through inhibition of GADD34 function.

Model



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Appendix I. Epidermal growth factor receptor-targeted therapy potentiates lovastatin-induced apoptosis in head and neck squamous cell carcinoma cells

Contribution

I performed the treatment of SCC25 cells with lovastatin and EGF and western blot analysis for Figure 6.

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ORIGINAL PAPER

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Epidermal growth factor receptor-targeted therapy potentiates lovastatin-induced apoptosis in head and neck squamous cell carcinoma cells

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Abstract *Purpose*: Mevalonate metabolites are vital for a variety of key cellular functions with the biosynthetic products including cholesterol and farnesyl and geranylgeranyl isoprenoids. Inhibition of this pathway using lovastatin induces a potent apoptotic response in a specific subset of human tumor-derived cell lines, including head and neck squamous cell carcinomas (HNSCC). In this study, we evaluated the potential of a number of chemotherapeutics that demonstrate activity in HNSCC, including an inhibitor of epidermal growth factor receptor (EGFR) to potentiate the cytotoxic effects of lovastatin. *Methods*: We evaluated the cytotoxic effects of combining a variety of chemotherapeutics with lovastatin using the MTT assay and flow cytometry. The MCF-7 lovastatin-resistant breast adenocarcinoma cell line and the lovastatin-sensitive HNSCC cell lines SCC9 and SCC25 were tested. Expression levels of EGFR and ligand activated EGFR following lovastatin treatment were analyzed by Western blotting. *Results*: Pretreatment or concomitant treatment of 10 μM lovastatin did not significantly augment the effects of a variety of chemotherapeutic agents tested in these cell lines. Co-administration with actinomycin D or cycloheximide, drugs that inhibit RNA and protein synthesis, respectively, inhibited lovastatin-induced apoptosis in these cell lines. This suggests a requirement for the cellular functions disrupted by these chemotherapeutic agents in lovastatin-induced apoptosis of HNSCC cells. In contrast to the chemotherapeutics analyzed, the AG1478 tyrosine kinase inhibitor of the EGFR demonstrated additive cytotoxic effects in combination with lovastatin in HNSCC cells. Mevalonate metabolites may regulate EGFR function, suggesting that lovastatin may inhibit the activity of this receptor. Indeed, lovastatin treatment inhibited EGF-induced autophosphorylation of the EGFR in the SCC9 and SCC25 cell lines. Pretreatment of SCC9 and SCC25 cell lines for 24 h with 10 μM lovastatin, conditions that demonstrated significant inhibition of EGF-induced EGFR autophosphorylation, induced significant additive effects in combination with AG1478. *Conclusion*: These results demonstrated the ability of EGFR pathway inhibitors to potentiate lovastatin-induced apoptosis and suggested that lovastatin may target the EGFR pathway in HNSCC cells.

Keywords Experimental therapeutics · Mevalonate pathway · HMG-CoA reductase · Epidermal growth factor receptor

Abbreviations *HNSCC* head and neck squamous cell carcinoma · *EGFR* epidermal growth factor receptor · *5-FU* 5-fluorouracil · *HMG-CoA* 3-hydroxy-3-methylglutaryl coenzyme A · *ActD* actinomycin D · *CHX* cycloheximide

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Introduction

HNSCCs include epithelial malignancies of the oral cavity, pharynx and larynx (Vokes et al. 1993). As a group, they represent the sixth most common human neoplasm, with an estimated annual worldwide incidence of 500,000 cases (Boring et al. 1992; Vokes et al. 1993). Despite significant advances in treatment using recent protocols for surgery, radiation and chemotherapy, the long-term survival of HNSCC patients has remained approximately 50% for the last 3 decades (Atula et al. 1997; Boring et al. 1994). Identification of active drugs in HNSCC and the optimization of scheduling and combination therapy has been a central component of these therapeutic approaches. Historically, the most active single agent in metastatic or recurrent HNSCC is cisplatin, which produces a

proper conformation and localization (Bishayee 2000; Slicker et al. 1988). Because of the potential of lovastatin to affect EGFR signaling through its ability to target HMG-CoA reductase, we also evaluated the effect of an EGFR tyrosine kinase inhibitor on lovastatin-induced apoptosis of HNSCC cells.

Materials and methods

Tissue culture

The SCC9 and SCC25 HNSCC and the MCF-7 breast adenocarcinoma cell lines were obtained from the ATCC (Rockville, Md.). The cell lines were maintained in Dulbeco's-MEM (Media Services, Ottawa Regional Cancer Centre) supplemented with 10% fetal bovine serum (Medicorp, Montreal). Cells were exposed to solvent control or to 0–100 μM lovastatin [generously provided by Apotex, Mississauga, Canada, diluted from a 10 mM stock in ethanol prepared as previously described, (Dimitroulakos and Yeger 1996)]. AG1478 (Calbiochem, San Diego) was diluted from a 50-mM stock in DMSO and human recombinant EGF (Sigma) was diluted from a 50-μg/ml stock in 10 mM acetic acid/0.1% bovine serum albumin (Sigma). The chemotherapeutics were obtained from the pharmacy at the Ottawa Regional Cancer Centre.

MTT assay

In a 96-well flat bottom plate (Nunc, Naperville, Ill.) approximately 5,000 cells/150 μl of cell suspension was used to seed each well. The cells were incubated overnight to allow for cell attachment and recovery. Following treatment, 50 μl of a 5 mg/ml solution in phosphate buffered saline of the MTT tetrazolium substrate (Sigma) was added and incubated for up to 6 h at 37°C. The resulting violet formazan precipitate was solubilized by the addition of 100 μl of a 0.01 M HCl/10% SDS (Sigma) solution shaking overnight at 37°C. The plates were then analyzed on an MRX Microplate Reader from Dynex Technologies at 570 nm to determine the optical density of the samples.

Flow cytometry

Cell cycle parameters were determined by flow cytometry using propidium iodide labeling of single cells as described previously (Dimitroulakos et al. 2001). Single cell suspensions were labeled with 50 μg/ml propidium iodide (Sigma) and approximately 10^6 cells in 100 μl analyzed by flow cytometry. Ten thousand cells were evaluated, and the percentage of cells in subG1 phase was determined using the Modfit LT program (Verity Software House, Topsham, Mass.).

Western blot analysis

Total cellular protein was extracted using a buffer that consisted of 1% Igepal CA-630 (Sigma), 0.5% sodium deoxycholate (Sigma), 0.1% SDS (Sigma), 0.2 mM sodium orthovanadate (Sigma) and 0.2 mM phenyl methyl sulphonyl fluoride (Sigma) in 2×PBS. Approximately 200 μl of extraction buffer was used to treat 10^6 cells. Total protein was quantified with the BioRad Protein Assay using bovine serum albumin (Sigma) as standard. Protein extracts representing 20 μg total protein from the cell lines and their treatments were separated on a 10% SDS-PAGE gel and electrophoretically transferred onto PVDF membranes (Amersham, Toronto). Membranes were blocked in 5% skim milk powder in PBS overnight at 4°C. Primary antibody, diluted in 5% skim milk powder in PBS, was incubated with the membrane for 1 h at room temperature. The polyclonal antibodies specific for EGFR and

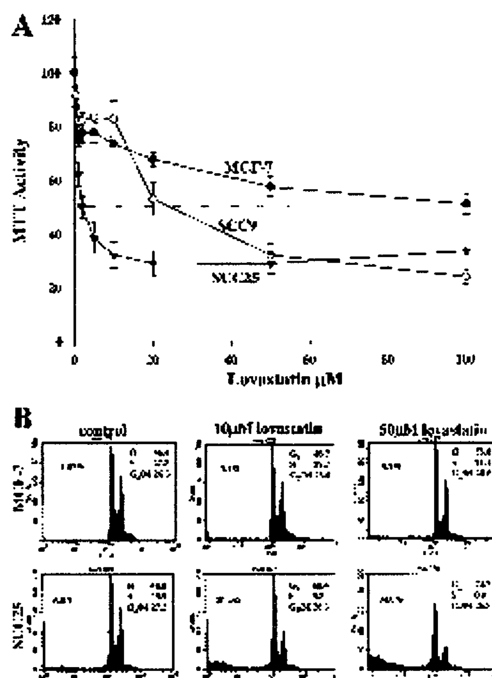

Fig. 1 Evaluating the effects of lovastatin on the viability of the MCF-7, SCC9 and SCC25 tumor-derived cell lines using MTT assay and flow cytometric analysis. **A** MTT enzyme activity after exposure to 0–100 μM lovastatin for 48 h, *highlighted* the three responses observed with this agent; non-responsive (*MCF-7*), intermediate (*SCC9*) and sensitive (*SCC25*). **B** Representative flow cytometric analyses of MCF-7 and SCC25 cell lines after exposure to solvent control, 10 μM or 50 μM lovastatin for 48 h. The percentage of cells in the subG1 (apoptotic) fraction is shown in the *upper left region* and the percentage of cells in each cell cycle phase is in the *upper right region* of each histogram. SCC25 cells displayed dramatic cell cycle and apoptotic responses to lovastatin exposure compared to MCF-7 cells

phospho-EGFR at site 1068 (Cell Signaling Technology, Beverly, Mass.) were used. The secondary antibody (Amersham) was applied at a 1:5,000 dilution in 5% skim milk powder in PBS and incubated for 1 h at room temperature (washes following antibody incubations are 3×5 min in PBS/0.05% Tween 80 (Sigma) then processed for chemiluminescent detection (Amersham). After the desired exposure was obtained the membrane was stained with Coomassie Blue (Sigma) to ensure equal loading of the samples.

Results

In this study, we focused on the potential of lovastatin to augment the cytotoxic effects of chemotherapeutic agents that have shown clinical activity in HNSCC. Due to the significant effects of cisplatin in HNSCC, we also included carboplatin and oxaliplatin in this

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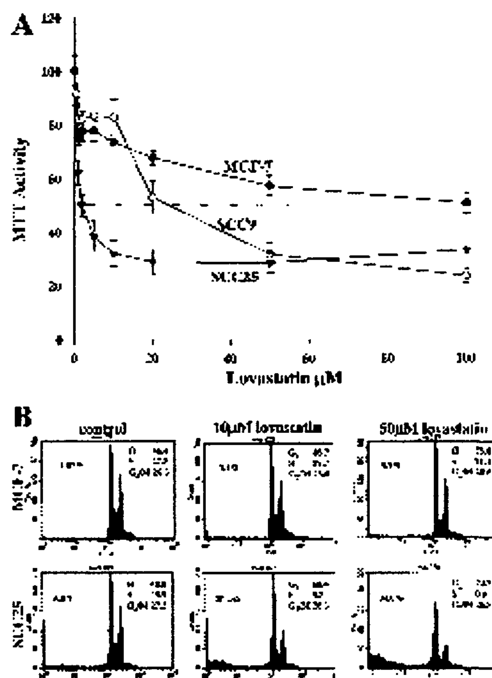

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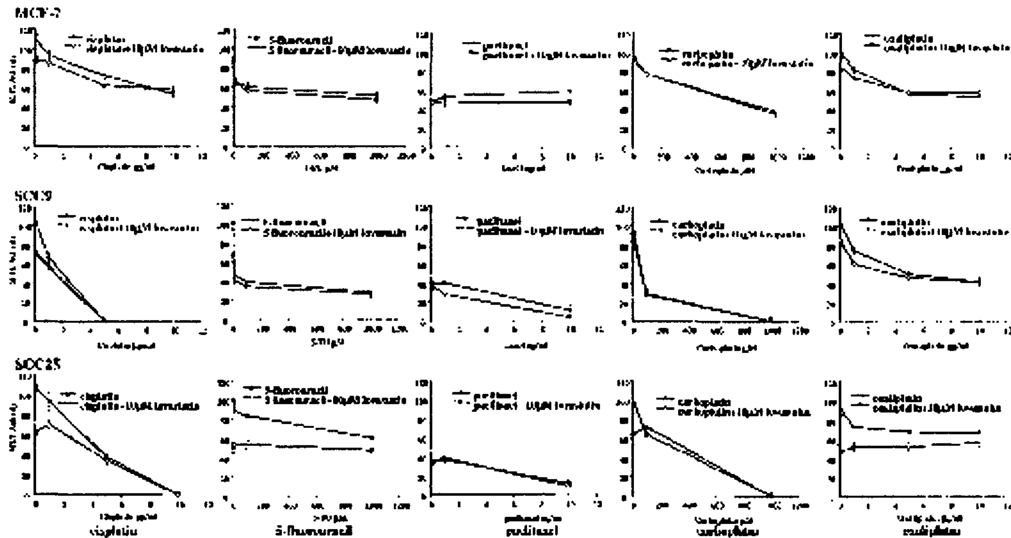


Fig. 2 Evaluating the effects on cell viability of combining 10 μM lovastatin with a variety of chemotherapeutics agents including cisplatin, 5-fluorouracil, paclitaxel, carboplatin and oxaliplatin. Cell viability was determined by MTT analysis following 48-h treatment with these combinations of agents. A variety of concentrations of each chemotherapeutic were analyzed alone or in combination with 10 μM lovastatin. No significant differences in responses were evident between either lovastatin and chemotherapeutic treatments alone and their combinations (paired *t*-test, data not shown)

study as they represent structurally distinct second and third generation platinum compounds, respectively (Kelland and McKeage 1994; Raymond et al. 1998). We compared the effects of combining cisplatin, 5-FU, paclitaxel, carboplatin and oxaliplatin in the HNSCC cell lines SCC9 and SCC25 as well as the breast adenocarcinoma cell line MCF-7 as a comparator. These cell lines represent the spectrum of sensitivity to lovastatin-induced cytotoxicity as MCF-7 cells are resistant, SCC9 display an intermediate response while SCC25 are sensitive to the apoptotic effects of this agent (Dimitroulakos et al. 1999) (Fig. 1A). The use of these three cell lines allowed the evaluation of the potential of lovastatin to augment the effects of traditional chemotherapeutics in cells that show differential sensitivities to lovastatin.

Flow cytometric analysis confirmed that this differential sensitivity was a result of significant growth inhibitory and apoptotic responses triggered in the sensitive SCC25 cell line that was not evident in MCF-7 cells under these experimental conditions (Fig. 1B). Cell cycle distribution and induction of apoptosis were visualized and quantified by determining cellular DNA content using propidium iodide staining (Darzynkiewicz et al. 1992; Piacentini et al. 1993). Apoptosis generally results in cellular and nuclear fragmentation with the formation of apoptotic bodies resulting in a subG1 population of cells (Darzynkiewicz et al. 1992; Piacentini et al. 1993). The S-phase fraction of SCC25 cells in cycle was dramatically reduced by lovastatin from 23.9% in untreated cells to 5.3% in cells treated with 10 μM for 48 h with a significant G1 cell cycle arrest from 48.8 to 68.4% in 10 μM 48-h treated cells (Fig. 1B). In this study, MCF-7 cells showed minimal cell cycle effects with this drug under these conditions. In the SCC25 cell line, lovastatin treatment also showed a significant percentage of cells in the apoptotic subG1 fraction that was not evident in the MCF-7 cell line. Lovastatin-induced apoptosis was significant at both 10 μM (20.4%) and 50 μM (34.0%) 48-h treatments (Fig. 1B). The SCC9 cell line is composed of a mixture of diploid and tetraploid cells and, although evident, evaluations of subG1 fractions due the complexity of the histogram were difficult to assess quantitatively using this method (data not shown).

Lovastatin does not significantly potentiate the cytotoxic effects of chemotherapeutics in HNSCC cells

To determine whether lovastatin can enhance the cytotoxicity induced by chemotherapeutic agents in HNSCC cells, we treated the MCF-7, SCC9 and SCC25 cell lines with 10 μM lovastatin in combination with a variety of standard chemotherapeutic agents. The concentration of lovastatin used is pharmacologically relevant as phase I clinical trials have demonstrated achievable serum concentrations of up to 4 μM sustainable over 7 days in cancer patients (Thibault et al. 1996). Various

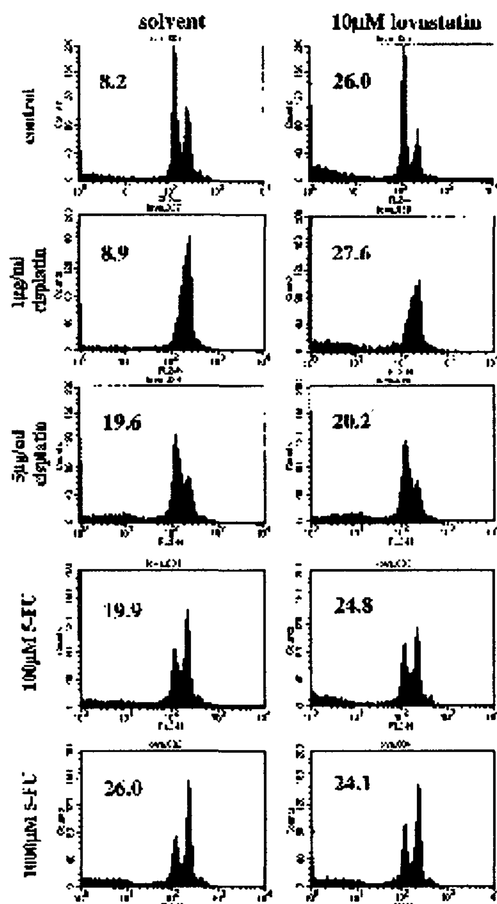


Fig. 3 Flow cytometric analysis of cisplatin and 5-FU-treated SCC25 cells alone or in combination with 10 μM lovastatin for 48 h. The subG1 (apoptotic) fraction of cells is displayed in the *upper left region* of the individual histograms. Combinations of either high or low concentrations of cisplatin or 5-FU did not potentiate the apoptotic effects of 10 μM lovastatin in SCC25 cells

concentrations of cisplatin, 5-FU, paclitaxel, carboplatin and oxaliplatin with or without the addition of lovastatin were evaluated by the MTT cytotoxicity assay following 48-h treatment (Fig. 2). The concentrations of these drugs and the time-point used was based on the NCI 60 cell line screen of these agents that assessed their sensitivity in a wide panel of human tumor-derived cell lines (Koutsoukos et al. 1994; Monks et al. 1997). In the MCF-7 cell line, lovastatin did not potentiate the cytotoxic effects of the drugs tested. There were no significant differences in cytotoxic effects when evaluating both lovastatin alone and drug alone versus their combination in all of the concentrations of drugs used (paired *t*-test analysis, data not shown). In the SCC9 cell line that shows intermediate sensitivity to lovastatin, borderline significant differences in both the lovastatin alone and drug alone versus their combination was limited to a single low dose of paclitaxel and oxaliplatin (paired *t*-test, data not shown). The SCC25 cell line that demonstrates sensitivity to lovastatin-induced apoptosis, did not demonstrate any significant differences in cytotoxicity when evaluating both lovastatin alone and drug alone versus their combination (paired *t*-test analysis, data not shown).

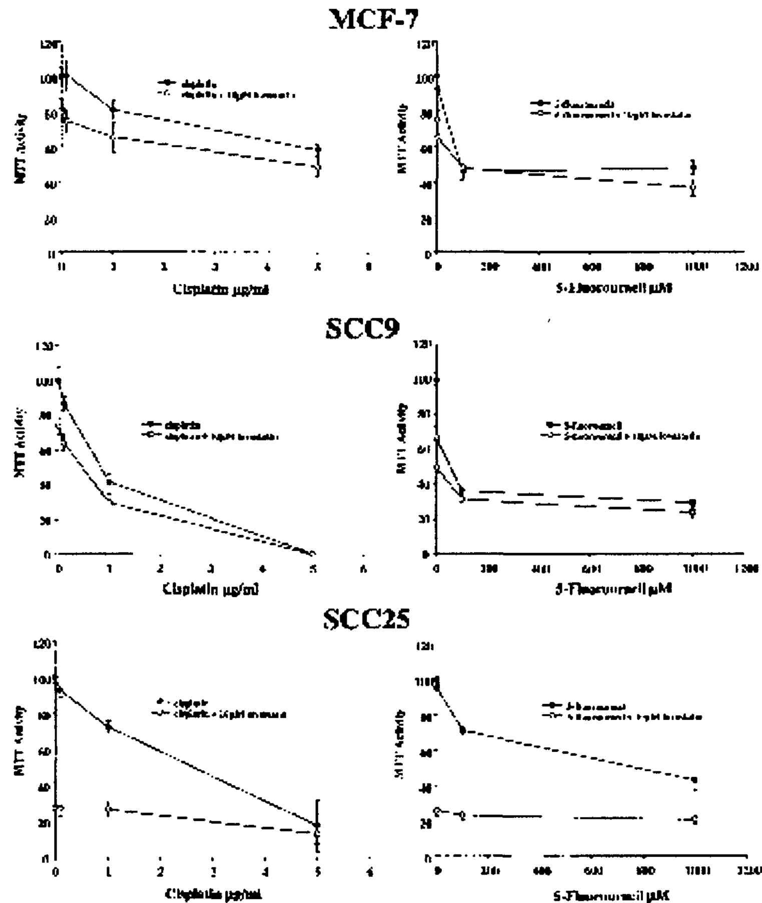
To evaluate this lack of co-operativity, the cell cycle and apoptotic effects of cisplatin and 5-FU ± lovastatin treatments for 48 h in the SCC25 cell line were analyzed by flow cytometric analysis. Based on their cytotoxic profiles determined by MTT analysis, both low and high concentrations of cisplatin (1 and 5 μg/ml) and 5-FU (100 and 1,000 μM) ± 10 μM lovastatin were evaluated (Fig. 3). Cisplatin induced a pronounced S-phase, while 5-FU induced a potent G2/M cell cycle arrest as has been previously reported for these agents (Cohen and Lippard 2001; Grem 2000). At the low concentrations of these agents, lovastatin-induced apoptosis was evident as prominent subG1 population of cells; however, the overall cytotoxicity as determined by MTT analysis is not enhanced by these combinations as the apoptotic fraction of cells remains at the levels of lovastatin treatment alone. At the high doses of the chemotherapeutics, lovastatin-induced apoptosis was inhibited and showed no augmentation of the apoptosis induced by cisplatin or 5-FU alone (Fig. 3).

In colon carcinoma cells, a recent report demonstrated that pretreatment with lovastatin for 48 h followed by combinations with cisplatin and 5-FU for an additional 48 h augmented lovastatin-induced apoptosis (Agarwal et al. 1999). These results were not additive but were more pronounced at concentrations of these chemotherapeutics that displayed weak cytotoxic responses (Agarwal et al. 1999). Similarly, pretreatment of MCF-7 and SCC9 with 10 μM lovastatin for 48 h followed by the combination with low dose cisplatin (0.1 and 1 μg/ml) for 48 h showed a weak augmentation of the cytotoxic effects of lovastatin (Fig. 4). No significant effects were demonstrated with lovastatin pretreatment in combination with 5-FU in these cell lines. The lovastatin-sensitive cell line SCC25, pretreatment with lovastatin followed by co-administration of cisplatin or 5-FU failed to demonstrate co-operativity (Fig. 4).

Actinomycin D and cycloheximide inhibit lovastatin-induced apoptosis

The lack of significant co-operativity between lovastatin and the chemotherapeutics tested in HNSCC cells suggests that the cellular functions disrupted by the cytotoxic doses of these chemotherapeutics may be required for lovastatin-induced apoptosis. In this study, we

Fig. 4 Evaluating the cytotoxic effects of a 48-h pretreatment of 10 μM lovastatin in combination with cisplatin and 5-fluorouracil. MCF-7, SCC9 and SCC25 cells were pretreated with solvent control or lovastatin followed by 48-h treatment with cisplatin or 5-flourouracil and cell viability determined by MTT assay. The lovastatin pretreatment was maintained through replenishment after the duration of the pretreatment. No significant additive effects with these chemotherapeutics were demonstrated in these cell lines with pretreatment with lovastatin



evaluated the requirement of transcription and translation in the apoptotic responses induced by lovastatin in HNSCC. Actinomycin D and cycloheximide are potent inhibitors of transcription and translation, respectively (Kim et al. 1998). Both agents have been used extensively to evaluate the roles of transcription and translation on biological processes. The MCF-7, SCC9 and SCC25 cell lines were treated with various concentrations of lovastatin with or without 5 ng/ml actinomycin D or 0.1 μM cycloheximide for 48 h and evaluated by MTT analysis (Fig. 5A). These concentrations of actinomycin D and cycloheximide have been shown to inhibit transcription and translation in a number of cell types including HNSCC cells (Kim et al. 1998). As expected, both of these drugs significantly inhibited MTT activity with actinomycin D demonstrating a weak apoptotic response (Fig. 5). In the MCF-7 and SCC9 cell lines, MTT activity of actinomycin D and cycloheximide remained relatively unchanged even with the addition of up to 50 μM lovastatin, indicating that these drugs inhibited the effects of lovastatin on these cells (Fig. 5A). In the lovastatin-sensitive SCC25 cell line, actinomycin D and cycloheximide protected against lovastatin-induced cytotoxicity as overall cell viability was increased in the combinations of agents as compared to lovastatin treatment alone. Flow cytometric analysis of these combinations in SCC25 cells showed an inhibition of lovastatin-induced apoptosis (Fig. 5B). These results suggest a requirement for these cellular functions to facilitate lovastatin-induced apoptosis.

Inhibition of EGFR potentiates lovastatin-induced apoptosis

A number of therapeutic approaches in HNSCC revolve around targeting and inhibiting the function of EGFR

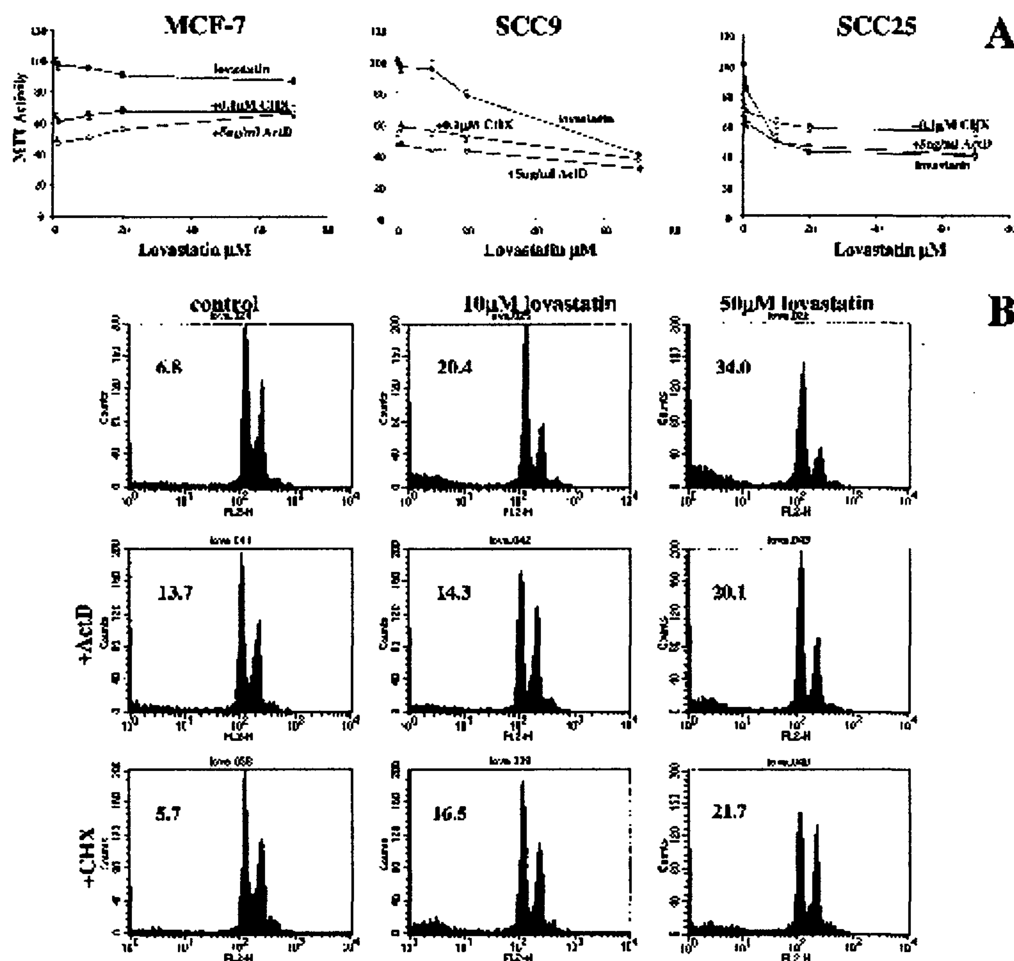


Fig. 5 Evaluating the effects of actinomycin D (*ActD*) and cycloheximide (*CHX*) on lovastatin-induced cytotoxicity and apoptosis in MCF-7, SCC9 and SCC25 cell lines. A MTT enzyme activity after exposure to 0–70 µM lovastatin alone or in combination with 0.5 ng/ml ActD or 0.1 µM CHX for 48 h was analyzed. The addition of ActD or CHX appeared to inhibit lovastatin-induced cytotoxicity in these cell lines; B flow cytometric analysis of solvent control, 10 µM lovastatin and 50 µM lovastatin with the addition of 5 ng/ml ActD or 0.1 µM CHX for 48 h was evaluated. Both *ActD* and *CHX* inhibited lovastatin-induced apoptosis in SCC25 cells

including blocking antibodies that inhibit ligand binding and specific tyrosine kinase inhibitors (Mendelsohn and Baselga 2000). Due to the essential role of mevalonate metabolites in transducing EGFR signals, in this study we evaluated the effects of the AG1478 EGFR tyrosine kinase inhibitor (Partik et al. 1999; Zhu et al. 2001) on lovastatin-induced apoptosis and the potential of lovastatin to target EGFR function. Using MTT analysis, AG1478-induced cytotoxicity was pronounced in the HNSCC cell lines SCC9 and SCC25, while the MCF-7 cells, where EGFR expression is not detected by Western blot analysis, showed toxicity only at high doses (200 µM) (Fig. 6A). At these doses, this drug can also affect other cellular targets (Partik et al. 1999; Zhu et al. 2001). Employing the 10-µM AG1478 concentration that did not affect MCF-7 MTT activity, this agent potentiated the cytotoxic effects of lovastatin in the SCC9 and SCC25 cell lines, but not the MCF-7 cell line (Fig. 6A). Although this effect was not additive, AG1478 was the only agent tested that consistently showed augmentation of lovastatin-induced cytotoxicity in our model systems.

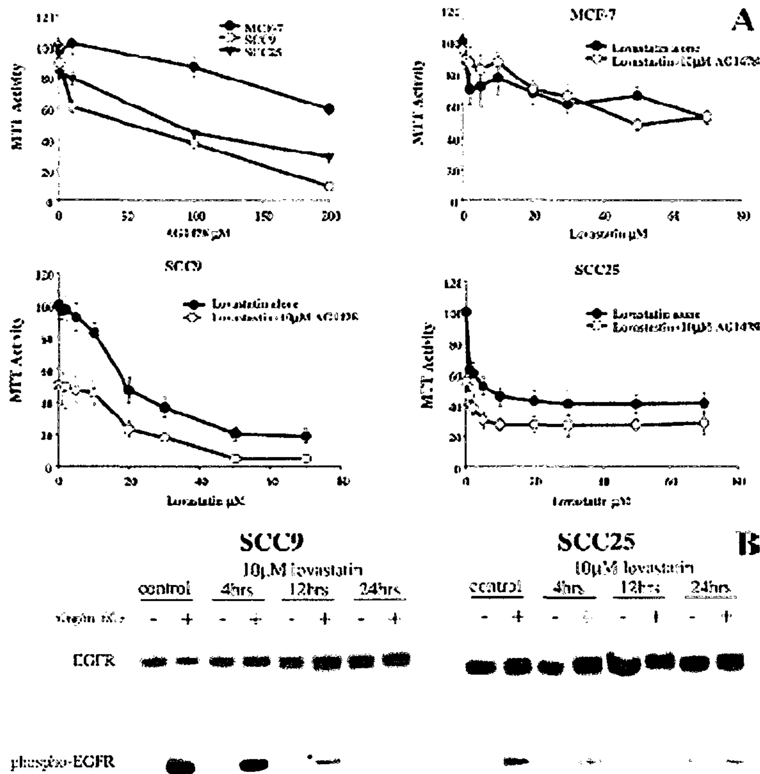


Fig. 6 A MTT analysis of AG1478 (EGFR tyrosine kinase inhibitor) exposure alone and in combination with lovastatin for 48 h. The SCC9 and SCC25 cell lines were sensitive to the cytotoxic effects of AG1478 in comparison to MCF-7. The combination of lovastatin and 10 μM AG1478 showed an additive effect in the SCC cell lines, but not in MCF-7. B Western blot analysis of EGFR and activated EGFR, visualized by the phospho-specific antibody that detects phospho-tyrosine at 1068 site of the receptor. Following addition of EGF for 15 min, the activation of EGFR was *inhibited* by lovastatin *in a* time-dependent manner. By 24 h treatment, the autophosphorylation of EGFR was abrogated. Therefore, lovastatin treatment appears to target the function of EGFR

The co-operativity of AG1478 and lovastatin may result from the ability of both of these agents to target EGFR signaling. We analyzed the ability of lovastatin to affect the activation of EGFR. In this experiment, we treated the SCC9 and SCC25 cell lines with 10 μM lovastatin for various time points up to 24 h with or without EGF addition to stimulate EGFR. Activated EGFR was visualized by Western blot analysis using a phosphospecific antibody that recognizes a phosphotyrosine at the 1068 amino acid site of this receptor (Bishayee et al. 1999) (Fig. 6B). Upon ligand stimulation, this site is autophosphorylated and is one of the main tyrosine phosphorylated residues that facilitates the transduction of signal from this receptor (Bishayee et al. 1999). Following 12- or 24-h treatment with lovastatin, EGF stimulated autophosphorylation of EGFR was significantly inhibited in both the SCC25 and SCC9 cell lines, respectively (Fig. 6B). The MCF-7 cells did not express detectable levels of EGFR or activated EGFR by Western blot analysis under these conditions (data not shown). Taken together, these results suggest that lovastatin may target EGFR function and that this may play a role in the anti-cancer properties of lovastatin.

Based on the observation that lovastatin inhibits autophosphorylation of EGFR in a time-dependent manner, we evaluated the effects of a 24-h pretreatment of these cell lines followed by combinations with AG1478. MTT analysis demonstrated a significant additive effect of this treatment regimen in SCC9 and SCC25 with no effect in MCF-7 cells (Fig. 7A). This combination induced a potent cytotoxic response in the HNSCC cells tested with a dramatic reduction of 80% in cell viability in the SCC9 and SCC25 cell with negligible effects on MCF-7 cells (Fig. 7A). Flow cytometric analysis showed that this additive cytotoxic effect was mediated by the ability of AG1478 to potentiate the apoptotic effects of lovastatin in the SCC25 cell line (Fig. 7B).

Discussion

Failure of cells to adequately control their proliferation, differentiation, cell survival and/or apoptosis contribute

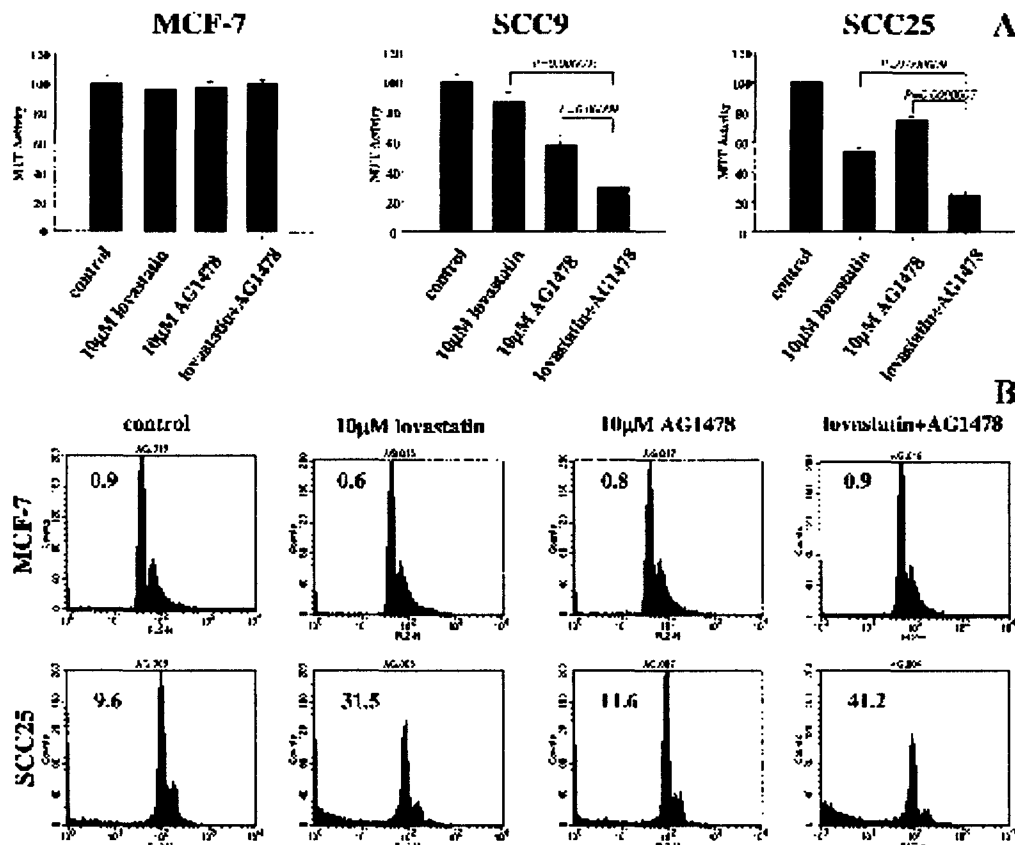


Fig. 7 MTT assay and flow cytometric analysis of MCF-7, SCC9 and SCC25 cell lines pretreated with either solvent control (control and 10 μM AG1478 treatments) or 10 μM lovastatin (10 μM lovastatin and lovastatin + AG1478 treatments) followed by 48-h treatment with lovastatin alone, AG1478 alone or their combination. The combination of lovastatin and AG1478 treatments demonstrated a significant additive effect compared to either treatment alone (paired *t*-test) in the SCC9 and SCC25 HNSCC cell lines, but not in the MCF-7 cells. Under similar conditions, flow cytometric analyses showed a marked potentiation of lovastatin-induced apoptosis with AG1478 with minimal effects on apoptosis or cell cycle in MCF-7 cells

to neoplastic transformation (Fisher 1994). Apoptosis is a highly regulated cellular process that can be activated as a result of aberrant proliferation or differentiation, abrogation of cell survival signals or in response to cellular damage resulting in programmed cell death (Fisher 1994). Chemotherapeutic agents and radiation target these cellular fates, particularly proliferation, resulting in the induction of an apoptotic response (Fisher 1994). More recently, novel agents and therapeutic approaches that regulate cell survival and signaling inducing apoptosis have been developed as a direct result in the progress of elucidating the cellular components of these pathways (Penn 2001). We recently identified lovastatin, a potent inhibitor of HMG-CoA reductase, as an agent that can trigger tumor-specific apoptosis, particularly in HNSCC derived cell lines (Dimitroulakos et al. 1999; Dimitroulakos et al. 2001; Dimitroulakos and Yeger 1996). Lovastatin is also a potent inhibitor of tumor cell growth (Keyomarsi et al. 1991) and as such has been evaluated as a potential anticancer therapeutic agent (Thibault et al. 1996). Based on these clinical studies, it has become apparent that the most relevant application of this agent would be as part of a combined modality approach.

In this study, we evaluated the potential of standard chemotherapeutics that have demonstrated activity in HNSCC to potentiate the apoptotic effects of lovastatin in preclinical in vitro studies. The chemotherapeutics tested did not significantly augment the apoptotic effects

of lovastatin in the HNSCC cell lines tested, and the higher cytotoxic levels of cisplatin and 5-FU apparently inhibited lovastatin-induced apoptosis. Furthermore, perturbing transcription or translation in these cells also inhibited lovastatin-induced apoptosis, suggesting that these cellular processes that are also affected by these chemotherapeutics (Cohen and Lippard 2001; Grem 2000) are required for the cytotoxic effects of lovastatin.

Lovastatin targets the rate-limiting enzyme of the mevalonate pathway, resulting in a depletion of its biosynthetic end products (Corsini et al. 1995; Hunninghake 1992). Mevalonate metabolites play significant roles in the post-translational modifications of a wide variety of key cellular proteins that are critical for their function. For example, dolichol is a molecular chaperone that modulates the N-linked glycosylation of a number of cell surface proteins including the EGFR (Bishayee 2000; Slicker et al. 1988). Glycosylation of EGFR is critical for its proper conformation and function as well as for efficient ligand binding and activation (Bishayee 2000; Slicker et al. 1988). As well, a wide variety of proteins are isoprenylated, the post-translational addition of a farnesyl or geranylgeranyl moiety that is required for their membrane localization and activity. Isoprenylated proteins include the members of the ras, rho and rab family of proteins (Gibbs et al. 1994; Pruitt and Der 2001; Seabra et al. 2002) that play key roles in transducing signals from a number of receptor tyrosine kinases including the EGFR (Gschwind et al. 2001; Pruitt and Der 2001; Takai et al. 2001). Therefore, inhibiting the mevalonate pathway has the potential to target EGFR and/or its signaling cascade.

The EGFR is a regulator of growth, differentiation and survival of epithelial cells and is also involved in the development and progression of cancers derived from these tissues, including HNSCC (Mendelsohn and Baselga 2000). Targeting EGFR function has been an intensive focus of anti-cancer therapeutic approaches in HNSCC (Arteaga and Johnson 2001; Mendelsohn and Baselga 2000). Due to the potential of statins to target EGFR downstream signaling and the availability of agents that target the EGFR more directly, we evaluated the potential synergy of combining these approaches. AG1478 is a potent and specific inhibitor of the EGFR tyrosine kinase, targeting the signal transduction pathway at the level of the receptor (Partik et al. 1999; Zhu et al. 2001). AG1478 demonstrated dramatic additive apoptotic effects with lovastatin in HNSCC cells. Furthermore, lovastatin displayed the potential to target EGFR function in a time-dependent manner, whereas AG1478 binds to the ATP binding site of this receptor directly inhibiting autophosphorylation (Partik et al. 1999; Zhu et al. 2001). These results indicate that these two therapeutic approaches may act co-operatively to target this receptor, albeit through different mechanisms. A number of therapeutic approaches targeting EGFR are currently in clinical trials, including ligand binding inhibitor antibodies (Mendelsohn and Baselga 2000) and various tyrosine kinase inhibitors of the receptor (Arteaga and Johnson 2001; Herbst 2002). The feasibility of combining statins with these clinically relevant EGFR inhibitors should be tested in well-established xenograft murine models of squamous cell carcinomas. These experiments were beyond the scope of this study. The combination of statins and EGFR tyrosine kinase inhibitors is an attractive therapeutic approach in HNSCC. Both agents alone demonstrate significant preclinical efficacy in HNSCC cells (Dimitroulakos et al. 2002; Dimitroulakos et al. 2001; Herbst 2002; Mendelsohn and Baselga 2000), and clinical trials have shown a different spectrum of toxicities (Arteaga and Johnson 2001; Herbst 2002; Thibault et al. 1996). Combining these therapeutic approaches will be invaluable in HNSCC patients. The ability of lovastatin to inhibit EGFR function is intriguing and requires further study to elucidate its mechanism and potentially allow for more refined therapeutic approaches using combinations of these agents.

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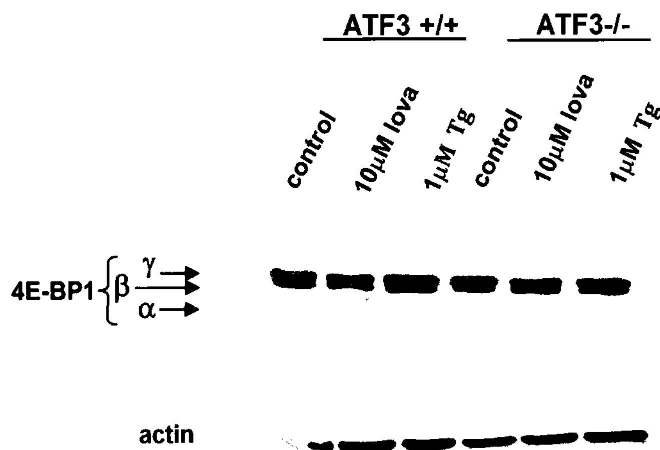
Appendix II.

Examining 4E-BP1 phosphorylation levels following lovastatin treatment in ATF3 null and wild-type MEFs. Cells were treated with vehicle (control), 10uM lovastatin, or 1ug TG for 24hr then total protein was extracted and analyzed by immunoblotting with anti-4E-BP1 antibody (Cell Signaling). Expression levels of actin were used as the loading control.

4E-BP1 is present in three isoforms which correspond to the different phosphorylation states of this protein. α and β forms are hypo-phosphorylated and represent active forms while the γ form is hyper-phosphorylated and is incapable of binding to and inhibiting transcription initiator eIF4E. Lovastatin increases the amount of inactive γ form of 4E-BP1 in both wt and ATF null cells.



Curriculum Vitae

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2. **Nima Niknejad**, Melissa Morley and Jim Dimitroulakos.Lovastatin Induced apoptosis triggered by the endoplasmic reticulum stress response. JBC , 2007.

3. Jennifer Li-Pook-Than, Catherine Carrillo, **Nima Niknejad**, Sophie Calixte, Jennifer Crosthwait and Linda Bonen .Relationship between RNA splicing and exon editing near intron junctions in wheat mitochondria. Physiologia Plantarum.2006.

4. Mantha AJ, McFee KE, **Niknejad N**, Goss G, Lorimer IA, Dimitroulakos J. Epidermal growth factor receptor-targeted therapy potentiates lovastatin-induced apoptosis in head and neck squamous cell carcinoma cells. J Cancer Res Clin Oncol. 2003 Nov;129(11):631-41

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2. **"Lovastatin induced apoptosis involves phosphorylation of eif2-alpha**

and induction of CHOP in squamous cell carcinoma" Nima Niknejad, Melissa Morley, Jim Dimitroulakos. Keystone meeting conference, Molecular Targets of Cancer Therapeutics meeting, March 2007, Whistler, BC, Canada

3. **"The role of ER-stress in Lovastatin induced apoptosis" Nima Niknejad**, Melissa Morley, Jim Dimitroulakos. AACR-NCI-EORT conference, Molecular Targets and Cancer Therapeutics meeting, November 2005, Philadelphia, USA
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2. Recipient of BMI travel award 2007
3. Recipient of BMI travel award 2008

LABORATORY SKILLS

Ottawa Regional Cancer Center:

1. Immunological assays
2. Western blotting
3. Flow cytometry
4. Tissue culture
5. Microarray analysis
6. Real-time PCR
7. Measuring rate of protein synthesis using S^{35} incorporation assay
8. Design and expression of stable small short hairpin interference RNA in mammalian system
9. Fluorescence microscopy
10. Measuring intracellular calcium levels
11. Cloning and expression of transient or stable exogenous gene in mammalian cells

Ottawa University:

1. Plant mitochondria DNA extraction
2. Plant mitochondria RNA extraction
3. Northern and Southern hybridization
4. PCR and RT-PCR (looking for very rare RNA transcripts)
5. Manual DNA sequencing on polyacrylamide gels
6. S1 nuclease assay, RNase protection assay

7. Cloning PCR products
8. Making evolutionary trees using SUN systems, strong bioinformatics background
9. Experienced in using molecular biology lab software eg. Li93, CODON, OMIGA

Shiraz University:

1. Western blotting
2. PCR