Resveratrol and Its Oligomers: Modulation of Sphingolipid Metabolism and Signaling in Disease

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Content

Abstract--Resveratrol, a natural compound endowed with multiple health-promoting effects has received much attention given its potential for the treatment of cardiovascular, inflammatory, neurodegenerative, metabolic and age-related diseases. However, the translational potential of resveratrol has been limited by its specificity, poor bioavailability and uncertain toxicity. In recent years, there has been an accumulation of evidence demonstrating that resveratrol modulates sphingolipid metabolism. Moreover, resveratrol forms higher order oligomers that exhibit better selectivity and potency in modulating sphingolipid metabolism. This review evaluates the evidence supporting the modulation of sphingolipid metabolism and signaling as a mechanism of action underlying the therapeutic efficacy of resveratrol and oligomers in diseases, such as cancer.

1. Introduction

First isolated in the 1940s, resveratrol (3,4′,5-trihydroxy-*trans*-stilbene) received little attention until 50 years later when it was found to be a major ingredient in red wine and identified as a chemo-preventive agent (Baur and Sinclair 2006). It is no surprise that resveratrol has been dubbed the "elixir of eternal youth" in view of its beneficial effects in preventing or slowing the progression of many human diseases by mediating cardiovascular protection, modulating lipoprotein metabolism and extending lifespan with its anti-inflammatory, anti-oxidant, anti-cancer and anti-aging properties (Orallo 2008). Despite advances in the field with evidence obtained from preclinical models, it is hard to comprehend how a simple molecule like resveratrol could act on a vast number of targets. Recently, several phytochemicals including resveratrol have been shown to inhibit membrane proteins non-specifically through perturbation of the lipid bilayer (Ingolfsson et al. 2014). In addition, many distal targets of resveratrol have been found but few studies have identified direct binding partners of resveratrol.

Using techniques such as X-ray crystallography, computer simulation and modeling, affinity chromatography, nuclear magnetic resonance studies, biochemical and biophysical analyses, several *bona fide* resveratrol binding targets have been characterized. These include sirtuin 1 (Sinclair and Guarente 2014), estrogen receptor α (Nwachukwu et al. 2014), cAMP phosphodiesterases (Park et al. 2012), cardiac protein troponin C (Pineda-Sanabria et al. 2011), leukotriene A_4 hydrolase (Oi et al. 2010), cyclooxygenase 1 and 2 (Szewczuk et al. 2004; Zykova et al. 2008), F1-ATPase (Gledhill et al. 2007) and quinine reductase 2 (Buryanovskyy et al. 2004). These biological targets link resveratrol to its pleiotropic effects such as lifespan extension, cardio-protection and chemoprevention. Another possibility for poly-pharmacology might be that, like a 'butterfly effect', resveratrol perturbs the activity of certain signaling molecules that regulate diverse functions in cellular homeostasis. Indeed, resveratrol is known to act on critical nodes in various signaling pathways including the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway (Widlund et al. 2013), estrogen signaling (Mobasheri and Shakibaei 2013; Signorelli and Ghidoni 2005), AMP-activated protein kinase/sirtuin 1/peroxisome proliferator-activated receptor gamma coactivator 1-alpha (AMPK/SIRT1/PGC-1α) pathway (Baur et al. 2012; Canto and Auwerx 2012) and stress-induced NF-κB signaling (Gupta et al. 2014; Wu et al. 2013).

Sphingolipids constitute one of the major classes of lipids in cells with diverse effects in regulating cellular processes, such as proliferation, survival, migration, differentiation and angiogenesis. Accumulating evidence suggests that some of the pharmacological activities of resveratrol are, in part, mediated through changes in sphingolipid metabolism. Moreover, resveratrol forms many bioactive oligomers, although these are less well characterized. This review aims to update the reader about the biological activity of resveratrol and to provide a current understanding of its effects on sphingolipid metabolism and signaling. Evidence will be presented for enhanced selectivity or potency of resveratrol oligomers. We will also discuss the therapeutic roles of resveratrol with respect to sphingolipid biology in various disease states.

1.1 Origin and activity of resveratrol oligomers

Resveratrol, produced in plants, belongs to a class of secondary metabolites known as stilbenoids that consist of two phenol rings linked together with an ethylene bridge (Fig. 1). The dietary source and activity of resveratrol have been extensively studied and reviewed (Baur and Sinclair 2006; Burns et al. 2002; Shukla and Singh 2011). Nevertheless, resveratrol can oligomerize to form oligostilbenoids in diverse plant families such as Dipterocarpaceae, Vitaceae, Leguminosae, Cyperaceae, and Gnetaceae (Sotheeswaran and Pasupathy 1993). Here we focus on resveratrol and its oligomers produced by plants in the Dipterocarpaceae, *Hopea* genus. Dipterocarpaceae is a well-known family of rainforest trees, which consist of approximately 500 species with greater than 100 species in the *Hopea* genus distributed mainly in tropical countries (Dayanandan et al. 1999). Selected resveratrol oligomers are presented in Fig. 1 to show the complexity achieved by natural oligomerization. A common biosynthetic route of these resveratrol oligomers has been proposed (Sotheeswaran and Pasupathy 1993) but their isolation from natural products has been a daunting task and it is not until recently that total synthesis has become feasible (Snyder et al. 2011).

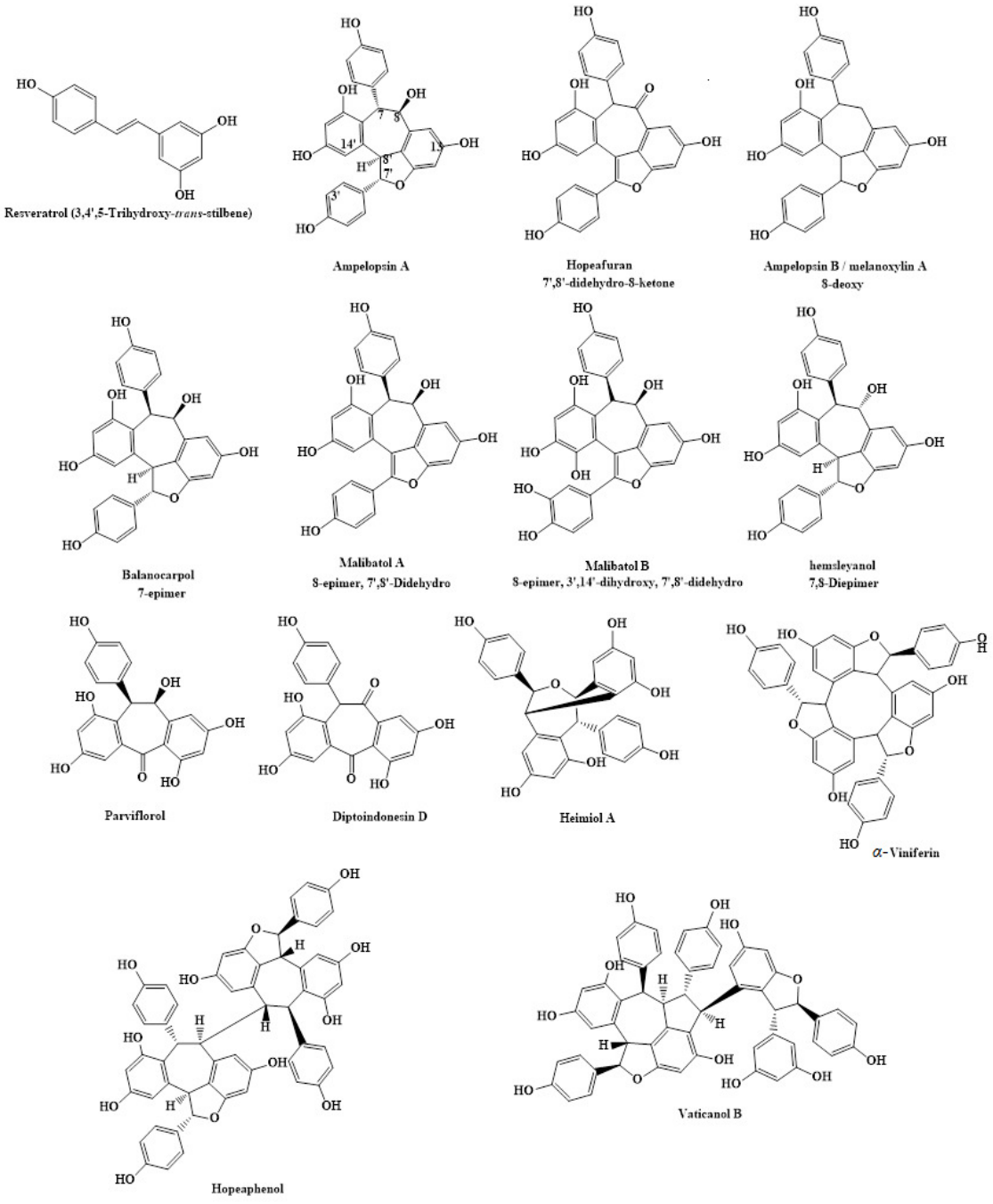

Figure 1. Representative structures of resveratrol and its oligomers isolated from *Hopea dryobalanoides*. Relative stereochemistry of each molecule is indicated.

Resveratrol tetramers

Hopeaphenol, a resveratrol tetramer, was one of the first compounds isolated from *Hopea odorata* and its structure confirmed by X-ray crystallography (Coggon et al. 1965; Coggon et al. 1966). Later, this compound was purified from other species in the same genus including *Hopea dryobalanoides*, *Hopea malibato* and *Hopea parviflora* (Dai et al. 1998; Sahidin et al. 2005; Tanaka et al. 2000). Interestingly, hopeaphenol has also been found in other plant genera including *Neobalanocarpus heimii*, *Dipterocarpus hasseltii* and *Vitis vinifera* (Muhtadi et al. 2006; Weber et al. 2001; Yan et al. 2001) suggesting its common existence as an important secondary metabolite. Hopeaphenol has strong growth inhibitory action against several cancer cells, such as human epidermoid nasopharynx carcinoma (KB), lung cancer carcinoma (A549), breast cancer (MCF-7) and murine leukaemia cells (P-388) (Muhtadi et al. 2006; Ohyama et al. 1999). Hopeaphenol also exhibits moderate anti-microbial activity against *Mycobacterium smegmatis* and methicillin-resistant *Staphylococcus aureus* (Zgoda-Pols et al. 2002). More recently, hopeaphenol has been found to block the type III secretion system essential for pathogenicity of gram negative bacteria, suggesting a selective inhibition of bacterial virulence (Zetterstrom et al. 2013). Taken together, hopeaphenol is one of the most active compounds produced by tropical trees. However, its mechanisms of action and direct protein targets remain to be elucidated. Vaticanol B is another resveratrol tetramer isolated from *Hopea dryobalanoides* and has been shown to moderately inhibit P-388 cell growth (Muhtadi et al. 2006; Sahidin et al. 2005). Vaticanol B has weaker growth inhibitory action compared with hopeaphenol, but exhibits anti-inflammatory effects that protect cells from ER-stress by inhibiting the activation of the unfolded protein response (UPR) genes (Tabata et al. 2007). Vaticanol C (an isomer of vaticanol B) induces apoptosis in various human cancer cell lines and a mouse mammary tumor model by perturbing mitochondrial membrane potential, activating pro-apoptotic proteins, such as caspases and Bad, down-regulating pro-survival signaling molecules, such as BCL2 and inhibiting ERK and Akt signaling pathways (Ito et al. 2002; Ito et al. 2003; Ohguchi et al. 2005; Shibata et al. 2007). Further investigations are needed to assess the pharmacokinetics of vaticanol C and potential toxicity in view of its therapeutic potential as an anti-cancer agent.

Resveratrol trimers

α-viniferin is a resveratrol trimer isolated from grapes of the common grape vine (*Vitis vinifera*) and exhibits anti-fungal activity (Langcake and Pryce 1977a). Interestingly, resveratrol, ε-viniferin (dimer) and α-viniferin (trimer) have been produced successively in a time-dependent manner under UV irradiation, indicating that the biosynthetic precursor is indeed, the resveratrol monomer (Langcake and Pryce 1977b). Similar to other resveratrol oligomers, α-viniferin inhibits cancer cell growth but fails to induce apoptosis of colon cancer cells (Gonzalez-Sarrias et al. 2011). α-viniferin suppresses interferon-γ-induced inflammation in mouse macrophages by down-regulating signal transducer and activators of transcription 1 (STAT1)-inducible inflammatory proteins, such as inducible NO synthase (iNOS), interferon-γ-inducible protein-10 (IP10) and monokine-induced by interferon-γ (MIG) (Chung et al. 2010). α-viniferin also reduces both early and late stages of LPS-induced inflammation in BV2 microglial cells through inhibition of PI3K/Akt-dependent NF-κB activation, reduced formation of pro-inflammatory molecules such as nitric oxide and prostaglandin E2 and suppression of nuclear factor erythroid 2–related factor 2 (Nrf2)- mediated haem oxygenase-1 expression (Dilshara et al. 2014). *In vitro* enzyme assays shows that α-viniferin has favorable anti-cholinesterase activity that might be useful for the treatment of Alzheimer's disease (Pinho et al. 2013; Sung et al. 2002; Yan et al. 2012). α-viniferin also exhibits activity against serotonin ($5\text{-}HT_6$) receptor (Kim et al. 2010), DNA topoisomerase II (Yamada et al. 2006), multidrug resistance-associated protein 1 (MRP1/ABCC1) (Bobrowska-Hagerstrand et al. 2006), COX-1 (Lee et al. 1998), COX-2 (Chung et al. 2003) and protein kinase C (Kulanthaivel et al. 1995; Xu et al. 1994). Together, these studies reveal an interesting insight into the mechanism of action of α-viniferin. This resveratrol trimer not only interacts with a large number of targets (estimated to be as diverse as resveratrol) but also has improved potency and may selectively access other biological targets with an "optimal" structure (see section 1.2 for discussion on resveratrol oligomerization). However, compared to the number of studies dedicated to resveratrol, fewer investigations have focused on α-viniferin. Future studies using animal models are needed to examine the physiological and therapeutic relevance of these targets since all studies cited above have only assessed α-viniferin *in vitro*.

Resveratrol dimers

Parviflorol, diptoindonesin D, balanocarpol, heimiol A and hopeafuran are examples of resveratrol dimers isolated from *Hopea dryobalanoides* (Sahidin et al. 2005). Parviflorol was first isolated from *Hopea parviflora* as a yellow solid and has moderate growth-inhibitory activity in cancer (Sahidin et al. 2005; Tanaka et al. 2000). Diptoindonesin D was subsequently isolated as a derivative (8-ketone) of parviflorol. Heimiol A was isolated as a light brown solid from *Neobalanocarpus heimii* (Weber et al. 2001). In fact, parviflorol and diptoindonesin D are modified dimers of resveratrol whereas balanocarpol and hopeafuran are derivatives of ampelopsin A (Fig. 1). Little information is available on the activities of these dimers. Notably, malibatol A and B isolated from *Hopea malibato* were found to be active against HIV in cultured human lymphoblastoid cells (Dai et al. 1998). Although balanocarpol shares similar structure with malibatol A and B, it has only modest activity against HIV. Kinetic inhibition studies establish that balanocarpol is a mixed competitive inhibitor (with sphingosine) of sphingosine kinase 1 (SK1) (Lim et al. 2012a). Despite successful isolation of resveratrol oligomers, further study has been hampered by low yield of these compounds and limited availability through total synthesis (Snyder et al. 2011). Conversely, resveratrol monomer is widely available and easily accessible; hence, is also extensively studied.

Resveratrol monomer

Several comprehensive reviews have been published concerning the *in vitro* and *in vivo* activities of resveratrol (Baur and Sinclair 2006; Signorelli and Ghidoni 2005; Widlund et al. 2013) . Therefore, a brief overview of its activity will be discussed here in relation to recent updates. Found abundantly in grapes and various dietary sources resveratrol has been isolated from over 70 plant species (Jang et al. 1997). Resveratrol is active against various human cancers as shown in cultured tumor cell and animal cancer models (Buryanovskyy et al. 2004). Anti-oxidant and anti-inflammatory effects were first proposed as the mechanisms for the anti-cancer and chemoprevention properties of resveratrol, which has phenol rings (strong scavengers of reactive oxygen species) and is able to inhibit COX-1 and COX-2 activities (Jang et al. 1997; Leonard et al. 2003; Subbaramaiah et al. 1998).

The anti-cancer activity of resveratrol has also been linked to perturbation of sphingolipid metabolism. In particular, resveratrol stimulates ceramide synthesis to induce apoptosis in breast cancer cells (Scarlatti et al. 2003). Resveratrol might also reduce the production of the pro-survival sphingolipid, sphingosine 1-phosphate (S1P). For example, resveratrol inhibits the activation of SK1 by phospholipase D and reduces SK1 expression to suppress pro-survival signaling in prostate cancer cells (Brizuela et al. 2010). Using an *in vitro* enzyme activity assay, SK1 has been found to be directly inhibited by resveratrol and balanocarpol (Lim et al. 2012a). Moreover, balanocarpol is two-fold more potent than resveratrol, suggesting that dimerization increases binding affinity for SK1 (see section 1.2 and Fig. 2). In addition to catalytic inhibition, both resveratrol and balanocarpol also down-regulate SK1 expression (Lim et al. 2012a). Therefore, resveratrol suppresses cell growth and induces apoptosis possibly by modulating the balance of ceramide and S1P in cancer cells.

Intense interests on resveratrol is due to its famous link with the "French paradox" where Mediterranean residents who consume red wine apparently have lower incidence of cardiovascular-related death, suggesting that resveratrol provides cardioprotection (Fremont 2000). However, a recent study that followed 783 elderly individuals who regularly consumed diets rich in resveratrol has found no significant association between any health benefits and total urinary resveratrol metabolite, casting doubts on its health-promoting effects (Semba et al. 2014). It is important to note that red wine (Guebailia et al. 2006) and other diets (Baechler et al. 2014; Barthomeuf et al. 2006) also contain significant amount of resveratrol oligomers that might be responsible for some of the beneficial health effects.

Notably, several plant polyphenols having similar structural moieties (e.g. piceatannol and resveratrol) were found to modulate life span and aging by activating sirtuin 1 (SIRT 1) (Howitz et al. 2003). The life span of yeast (*Saccharomyces cerevisiae*) was extended by 70% at low micromolar concentrations of resveratrol and attributed to activation of sirtuins that stabilize ribosomal DNA repeats rather than its anti-oxidant activity (Howitz et al. 2003). Subsequently, resveratrol has been shown to have similar effects as calorie restriction in prolonging the lives of roundworms, fish, flies, bees and mice (fed on a high-fat diet), indicating an evolutionary conserved mechanism of SIRT1 in regulation of health and lifespan (Baur et al. 2012; Sinclair and Guarente 2014). However, resveratrol-induced activation of SIRT1 and its role in

prolonging lifespan is controversial with divergent views on whether this is a direct effect. *In vitro* assays measuring the activation of SIRT1 by resveratrol has been found to be dependent on fluorogenic substrates, raising doubts on the robustness of the assay (Borra et al. 2005; Kaeberlein et al. 2005). A recent study by Park and colleagues also supports an indirect effect of resveratrol via inhibition of cAMP phosphodiesterases leading to activation of the AMPK/SIRT1 signaling pathway (Park et al. 2012). Despite these findings, resveratrol has been shown to bind to and activate SIRT1 directly via an allosteric mechanism (Hubbard et al. 2013), indicating that the anti-aging effect of resveratrol might be SIRT1-dependent (Sinclair and Guarente 2014). A subset of low molecular weight chemicals with related stilbene scaffolds can also activate SIRT1 (Howitz et al. 2003). Since resveratrol oligomers might have similar targets, it will be of interest to investigate whether resveratrol oligomers can activate SIRT1 and mimic life-extending and health-promoting effects of calorie restriction.

1.2 Resveratrol oligomerization

Why would nature produce structurally diverse resveratrol oligomers? There are several possible explanations when looking at the evolution of conserved biosynthetic pathways. Indeed, two models have been proposed for the evolution of structurally-related natural compounds: target-based and diversity-based models (Fischbach and Clardy 2007). The first model consists of secondary metabolites that show high affinity for their respective biological targets and these molecules have evolved to improve host survival. For example, rapamycin (a polyketide) is an immunosuppressant that specifically inhibits FK-binding protein (FKBP-12) and mammalian target of rapamycin (mTOR) by forming a FKBP-12-rapamycin-mTOR complex (Choi et al. 1996). This elegant work demonstrates that rapamycin functions as a 'molecular glue' that simultaneously blocks downstream signaling of two different proteins. The second model posits that evolution favors the survival of organisms that can maximize the diversity of their secondary metabolites. For example, terpenes consist of a large group of organic compounds, all derived from a simple building block, the isoprene (C_5H_8). At least 136 distinct gibberellin-family diterpenes are found in nature but only a few of them have potent biological effects; most are regarded as side products that lack activity (Fischbach and Clardy 2007). Therefore, certain secondary metabolites are synthesized in a diversity-oriented approach to maximize diversity and minimize metabolic cost by utilizing a common building block. In

common with terpenes, resveratrol oligomerization is regarded as a diversity-oriented approach and evidence will be provided in the following to support this possibility.

Resveratrol oligomerization possibly arises through the need for improved potency and selectivity. Qiao and colleagues screened 31 resveratrol oligomers and found that several trimers and one tetramer are more effective than the monomers or dimers in suppressing growth of several human cancer cell lines (Table 1). These workers investigated the anti-tumor efficacy of pauciflorol B (trimer) in a murine tumor model and established p53-dependent apoptosis and cell senescence as the mechanisms of action (Qiao et al. 2013). Similarly, the rank of potency for growth inhibition in P-388 murine cancer cells is hopeaphenol (tetramer) > α-viniferin (trimer) > balanocarpol (dimer) (Sahidin et al. 2005). In addition, ε-viniferin (dimer) and miyabenol C (trimer) induced apoptosis in human myeloma U266 cells at lower concentrations than resveratrol (Barjot et al. 2007). Vaticanol C (tetramer) is also 4-7 fold more potent than resveratrol in killing SW480 and HL60 colon cancer cells (Ito et al. 2003). The anti-inflammatory activity is also increased with oligomerization since, vaticanol B (tetramer) is more active than resveratrol monomer and dimer (Tabata et al. 2007). Taken together, oligomerization improves the anti-cancer properties of resveratrol in terms of growth suppression and apoptosis.

Other cellular targets such as DNA topoisomerase II have been found to be more effectively inhibited by resveratrol oligomers (e.g. hopeaphenol) compared with the resveratrol monomer (Baechler et al. 2014). Interestingly, molecular size does not seem to be a constraint on the potency of higher order resveratrol oligomers since resveratrol hexamer still retains comparable activity compared with other tetramers against DNA topoisomerase II (Yamada et al. 2006). In the latter study, oligomers with higher repeating resveratrol units are also more active than smaller molecules (Table 1). In contrast, resveratrol is the most potent compound in inhibiting tyrosinase (an oxidase mediating the production of melanin) whereas modification of resveratrol by glucosidation (e.g. piceid), reduction (e.g. dihydroresveratrol) and oligomerization (e.g. α-viniferin) greatly reduces or abolishes activity against tyrosinase (Ohguchi et al. 2003). α-viniferin (trimer) inhibits cholinesterase (implicated in Alzheimer's disease) whereas resveratrol is not active at concentrations up to 500μM (Pinho et al. 2013). Moreover, α-viniferin (trimer) is the most potent inhibitor of cholinesterase compared with the tetramer, kobophenol A (Sung et al. 2002). In addition, α-viniferin is

also 3-4-fold more potent than resveratrol at reducing prostaglandin H2 activity (Lee et al. 1998). α-viniferin is also the most potent compound in blocking MRP1/ABCC1 activity whereas resveratrol is inactive (Bobrowska-Hagerstrand et al. 2006), indicating that some enzymes and proteins have strict steric and conformational requirements for resveratrol oligomers.

Another target of resveratrol oligomers is SK1, which catalyzes the phosphorylation of sphingosine to produce the bioactive lipid, S1P. S1P maintains cell survival and growth (Pyne and Pyne 2000) and is also involved in plant signaling (e.g. transpiration and seed germination) (Worrall et al. 2008). We found that balanocarpol (dimer) is more potent in inhibiting SK1 with an inhibition constant (K_i) of 90 ±10μM compared with that for resveratrol of 160 ± 40μM. These findings suggest that dimerization increases potency against SK1 (Lim et al. 2012a). The molecular interactions of resveratrol and balanocarpol with SK1; the crystal structure having been recently resolved (Wang et al. 2013) have now been modeled by us (Fig. 2). The predicted binding mode of resveratrol shows that its hydroxyl groups forms hydrogen bonds with the backbone carbonyl of L268 and L299 and one of the side chain oxygens of D178 (the deprotonating base enabling nucleophilic attack by sphingosine on the γ-phosphate group of ATP) in the catalytic site of SK1. Due to increased size and number of hydroxyl groups (six in total), balanocarpol is modelled to bind to L268 and D178 but can form additional hydrogen bonds with the side chain oxygen of T196, the second carboxylate oxygen of D178 and the backbone carbonyl of A262. These modelling data can therefore provide an explanation for the increased potency of balanocarpol compared with resveratrol in inhibiting SK1 activity.

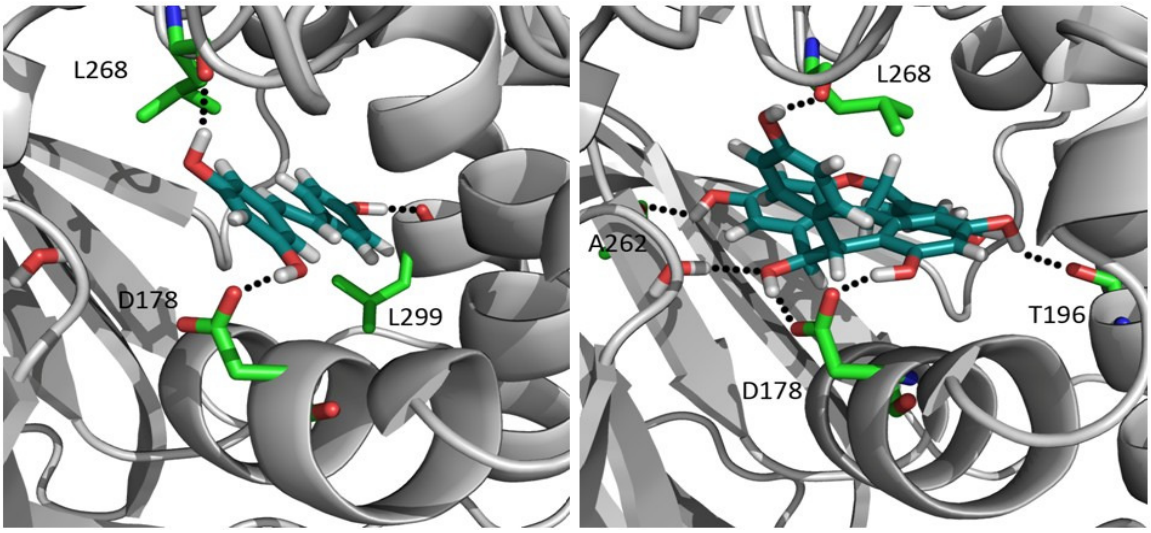

Figure 2. Binding modes obtained by docking resveratrol (left) and balanocarpol (right) in the sphingosine binding site of SK1 (PDB entry 3VZB). Chain A of the crystal structure of SK1 in complex with sphingosine was used to dock resveratrol and balanocarpol. The water molecule found to be tightly bound to the side chain –OH of S168, the backbone –NH of G342 and the secondary hydroxyl group of sphingosine is included in the modelling. Both compounds were docked using GOLD 5.2 for Windows (Cambridge Crystallographic Data Centre, Cambridge, UK), using default parameters and allowing the side chains of L259, L261, L263 and L302 to be freely flexible during the study. The flexibility of the side-chains of these leucines was required to allow the sphingosine binding site of SK1 to accommodate the bulky balanocarpol.

A distinct inhibitory profile has been documented for vitisin A and hopeaphenol, both of which are tetrameric forms of resveratrol. Unlike hopeahenol that consists of two repeating resveratrol dimers (ampelopsin B), vitisin A is a complex of one ε-viniferin and one ampelopsin B. Opposing effects were observed for vitisin A (pro-apoptotic) and hopeaphenol (anti-apoptotic) on calcium-induced cytochrome C release and mitochondrial depolarization in cardiac myocytes (Seya et al. 2009). Peroxisome proliferator-activated receptors (PPAR, nuclear receptors that act as transcription factors) are also activated by resveratrol and vaticanol C (tetramer) but not ε-viniferin (dimer) (Tsukamoto et al. 2010). In addition, vaticanol C binds $PPAR_{\alpha}$ and $PPAR_{\beta/\delta,}$ yet resveratrol stimulates all isoforms of PPAR. Interestingly, vaticanol C or ε-viniferin do not activate SIRT1 (Tsukamoto et al. 2010). Overall, these findings suggest that resveratrol oligomerization improves binding affinity (potency) and enhances target selectivity.

Polyphenols produced by plants might be used by animals as a signaling cue to improve survival when confronted by environmental stress (Howitz and Sinclair 2008). This hypothesis (termed xenohormesis) was supported by observations that a wide range of natural compounds interact with different signaling pathways in animals. In fact, the similarity between many signaling molecules in plants and animals indicates that common biosynthetic pathways existed before the two kingdoms diverged (Kushiro et al. 2003). A large amount of research over the past 20 years has shown that resveratrol not only serves specific roles in plant development and defense but can also target diverse signaling pathways in animals. The myriad biological functions of resveratrol might be explained by its simple, yet unique structure that has

survived evolution. Thus, oligomerization provides a means to achieve better selectivity and potency to minimize metabolic cost. Further investigations of these compounds should provide impetus to fully develop the therapeutic potential of resveratrol.

Table 1. Relative efficacies of representative resveratrol oligomers for different signaling molecules and pathways

Signaling pathway (Cell line or biological target)	Monomer (IC_{50} µM)	Dimer (IC_{50} µM)	Trimer (IC_{50} µM)	Tetramer (EC/IC_{50} µM)	References
Apoptosis (SW480)	resveratrol (22.1)	ε-viniferin (>100)	α-viniferin (18.5)	vaticanol C (3.6) hopeaphenol (28.6)	(Ito et al. 2003)
Apoptosis (HL60)	resveratrol (13.1)	ε-viniferin (44.2)	α-viniferin (5.2)	vaticanol C (3.0) hopeaphenol (21.3)	
Chromosome condensation (DNA topoisomerase II)	resveratrol (262)	balanocarpol (47)	α-viniferin (27) α-viniferin 13-*O*-*β*-glucopryranoside (4)	hemsleyanol C (1)	(Yamada et al. 2006)
Cholinergic neurotransmission (Cholinesterase)	resveratrol (>5)	NA	α-viniferin (2)	kobophenol A (115.8)	(Pinho et al. 2013; Sung et al. 2002)
Drug resistance (MRP1)	resveratrol (NE)	ε-viniferin (8.9)	α-viniferin (0.8)	NA	(Bobrowska-Hagerstrand et al. 2006)
ER stress (F9 Herp-null)	resveratrol (NE)	ε-viniferin (NE)	NA	vaticanol B (~5-10)	(Tabata et al. 2007)
Gene transcription (PPARα)	resveratrol (5)	ε-viniferin (NE)	NA	vaticanol C (2.5)	(Tsukamoto et al. 2010)
Growth inhibition (P-388)	NA	balanocarpol (33.6)	α-viniferin (25.8)	hopeaphenol (5.7)	(Sahidin et al. 2005)
Growth inhibition (MCF7)	resveratrol (>70)	parviflorol (>30)	pauciflorol B (5.0)	vaticaffinol (9.6)	(Qiao et al. 2013)
Growth inhibition (MDA-MB-231)	resveratrol (>70)	parviflorol (>30)	pauciflorol B (17.7)	vaticaffinol (26.3)	
Growth inhibition (MCF7)	resveratrol (50)	ε-viniferin (10)	NA	NA	(Lim et al. 2012a)
Growth and survival (SK1)	resveratrol (160)*	ε-viniferin (90)*	NA	NA	

* values indicate inhibition constant (K_i); NA, Not Available; NE, Not Effective; ER, Endoplasmic Reticulum; MRP1, Multidrug Resistance-Associated Protein 1; PPAR α, Peroxisome Proliferator-Activated Receptor α

1.3 Pharmacokinetics and toxicity

One of the main obstacles in translating the beneficial effects of resveratrol to the clinic is its poor pharmacokinetic profile. Various studies have documented the bioavailability of resveratrol in human and animals. Resveratrol is quickly metabolized in the body to sulphate and glucuronide conjugates within 30 minutes of intravenous administration; the half-lives of resveratrol and total resveratrol metabolite is 8-14 minutes and approximately 9 hours respectively (Baur and Sinclair 2006). Notably, resveratrol sulphate is actively taken up by cells and provides a reservoir of intracellular resveratrol. Peak plasma and local tissues concentration of resveratrol sulphate after oral dosing of 1g daily is 30 and 640 μM respectively (Patel et al. 2013). Resveratrol is delivered to cells as a sulphate conjugate that is further metabolized by the cells to regenerate resveratrol. Therefore, bioavailability of resveratrol and its route of administration/delivery system may not be a major concern.

Toxicity is an issue that requires consideration. This is especially true for resveratrol, which has to be administered at high dosage to achieve clinical response including anti-cancer effects. However, resveratrol does not appear to have any detrimental effects in rats at 300mg/kg. Toxicity in man is less well studied or documented due to multiple challenges associated with human clinical trials (Smoliga et al. 2012). Very recently, resveratrol administered to non-human primates was found to exhibit several health benefits as observed from rodent studies. However, an abnormal developmental effect was found in fetal pancreas, arguing against the use of resveratrol in pregnant woman (Roberts et al. 2014). Resveratrol might also interact with other dietary supplements or drugs as it inhibits human cytochrome P450 (CYP) enzymes (Baur and Sinclair 2006). α-viniferin also inhibits 7 out of 9 human CYP isoforms that are involved in drug metabolism, indicating the potential for drug interactions (Sim et al. 2014). Consequently, toxicity and side effects of resveratrol and its oligomers should be thoroughly examined before they can be recommended for long-term use.

2. Sphingolipids

Lipids (glycerolipids, sphingolipids and sterols) are ubiquitous biomolecules that are involved in the regulation of cellular homeostasis in health and disease. Bioactive sphingolipids play important roles in mammalian cell signaling (Hannun and Obeid 2008). The name of sphingolipids is derived from the mystical "sphinx", which suggests their enigmatic properties. For decades, sphingolipids have been considered as inert structural components of the cell membranes. Sphingolipids have come of age and have emerged as pleiotropic signaling molecules that regulate various cellular processes such as apoptosis (Hannun and Bell 1989). Sphingolipids also stabilize lipid microdomains (or "lipid rafts") where cell signaling is compartmentalized and facilitated by the biophysical functions of sphingolipids (Futerman and Hannun 2004). Certain molecular species of ceramide induce apoptosis (Obeid et al. 1993) whereas sphingosine-1-phosphate (S1P) promotes cell proliferation (Olivera and Spiegel 1993), prevents apoptosis (Cuvillier et al. 1996) and functions as a ligand for five G-protein coupled receptors (named $S1P_{1\text{-}5}$). Moreover, S1P signaling plays a major role in many human diseases including cancers (Pyne and Pyne 2010) and immune disorders (Pyne and Pyne 2011). Furthermore, the sphingolipid pathway has been successfully targeted by Fingolimod (FTY720), the first FDA-approved drug modulating the $S1P_1$ receptor and now licensed as Gilenya® for the treatment of relapsing-remitting multiple sclerosis (Brinkmann et al. 2010). In recent years, compounds such as RPC1063 and ponesimod (ACT-128800) that selectively target the $S1P_1$ receptor have entered clinical trials for the treatment of autoimmune diseases including multiple sclerosis, psoriasis and ulcerative colitis. Moreover, the S1P specific monoclonal antibody, Sonepcizumab ('S-one-P' cizumab) or LT1009 has been developed into two different formulations for clinical trials: (i) ASONEP™ for non-resectable and refractory kidney cancer; and (ii) iSONEP™ for age-related macular degeneration. These clinical evaluations highlight the therapeutic potential of modulating S1P signaling in chronic inflammatory diseases (Schwalm et al. 2014).

2.1 Sphingolipid Metabolism

There are a multitude of lipids with distinct structures that are present in cells (Futerman and Hannun 2004). This structural diversity and complexity is the result of the availability of diverse substrates for lipid-metabolizing enzymes. With the advent of molecular cloning and biochemical analysis, the sphingolipid

metabolic pathways have been elucidated. The neutral building block of all sphingolipids is ceramide that is either formed by *de novo* synthesis at the endoplasmic reticulum (ER) or activation of sphingomyelinases which function in the so-called 'salvage pathway'. In the ER, serine and pamitoyl coenzyme A is condensed by serine palmitoyl transferase (SPT) to form 3-ketosphinganine that is reduced to dihydrosphingosine followed by N-acylation to form dihydroceramide. In turn, dihydroceramide can be desaturated to form ceramide (Fig. 3). SPT has recently been shown to be negatively regulated by the Orm proteins that are encoded by the *ORMDL* genes in human (Breslow et al. 2010). Through genome wide association studies, *ORMDL3* gene has been implicated as a significant risk factor for childhood asthma, primary biliary cirrhosis, diabetes and Crohn's disease. These findings suggest that disruption of sphingolipid homeostasis could play a role in pathogenesis of these inflammatory diseases (Breslow et al. 2010). Sphingomyelinases catalyze the hydrolysis of sphingomyelin that is abundant in the cellular membrane to produce ceramide in the sphingosine salvage pathway. Sphingomyelinases are broadly categorized in neutral, acid or alkaline forms. Ionizing radiation activates acid sphingomyelinase to produce ceramide which promotes the induction of apoptosis (Haimovitz-Friedman et al. 1994), implicating a crucial role for this enzyme in mediating stress-induced apoptosis (Santana et al. 1996). Conversely, a cell permeable and non-competitive inhibitor of neutral sphingomyelinase (GW4869) blocks ceramide synthesis to rescue cell death in numerous cell types, such as cochlear hair cells (Chi et al. 2014), retinal pigment epithelial cells (Kucuksayan et al. 2014) and fibroblasts (Qin et al. 2012). At least four isoforms of neutral sphingomyelinase are expressed in mammalian cells, each with distinct biochemical properties, localizations and physiological roles (for a review, see Airola and Hannun 2013)). Alkaline sphingomyelinases are less well studied. These enzymes are expressed mainly in the intestines and could be implicated in various digestive tract diseases (Duan 2006). Together, these studies provide a strong rationale for therapeutic targeting of sphingomyelinases to modulate the level of ceramide in various diseases.

Ceramide can be deacylated (removal of the fatty acid side chain) through a reaction catalyzed by ceramidase. Conversely, sphingosine can be acylated by ceramide synthase to produce ceramide. Phosphorylation of sphingosine is catalyzed by sphingosine kinase (two isoforms termed SK1 and SK2) to produce S1P, which can be recycled to sphingosine by lipid phosphate phosphatases or S1P specific

phosphatases. Alternatively, S1P is cleaved by S1P lyase to produce phosphoethanolamine and (*E*)-2-hexadecenal and this represents the only exit point in the sphingolipid metabolic pathway (Fig. 3).

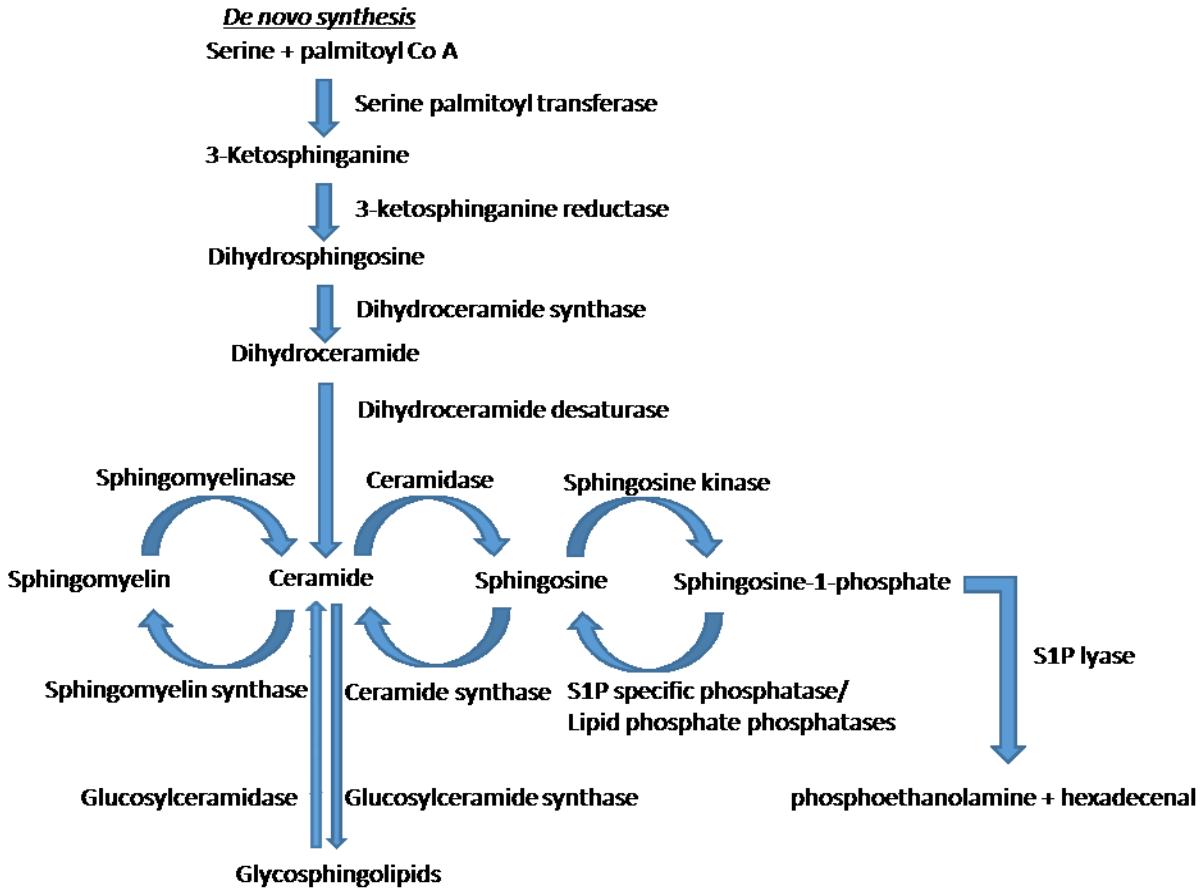

Figure 3. Sphingolipid metabolism. Degradation of sphingomyelin or *de novo* synthesis produces ceramide that is the precursor of sphingosine, sphingomyelin and glycosphingolipids. Sphingosine is phosphorylated by sphingosine kinase to form sphingosine-1-phosphate (S1P) which can be recycled back to sphingosine by enzymes like S1P specific phosphatases. S1P is irreversibly cleaved by S1P lyase to exit the sphingolipid metabolic pathway.

Understanding the metabolism of sphingolipids is essential to dissect the mechanisms of action for agents that perturb sphingolipid levels. In addition, these metabolic pathways are bi-directional and sphingolipid levels are tightly regulated in cells. For example, the apparent failure of a ceramide-inducing agent to promote apoptosis might be due to an active clearance of ceramide by ceramidase. Indeed, ceramidase

inhibitors might be usefully exploited to reduce ceramide clearance and hence potentiate ceramide-dependent apoptosis (Canals et al. 2011). In diseased states, one or more of these metabolic pathways might be deregulated resulting in over-production of a metabolite; for example, by over-expression of the anabolic enzymes or down-regulation of the catabolic enzymes. Indeed, S1P levels are increased by elevated expression of SK1 in multiple human cancers (Pyne and Pyne 2010). In addition, down-regulation of alkaline sphingomyelinase has been observed in human colon and liver cancers leading to reduction of ceramide level and attenuation of apoptosis (Cheng et al. 2007; Duan 2005). Therefore, the interconversion of these sphingolipid metabolites must be considered when assessing the cellular phenotype upon gene knockdown or treatment with pharmacological therapeutic agents.

2.2 Biological activity of sphingolipids

The sphingolipid 'rheostat' has been proposed as a model to explain the biological activity of ceramide, sphingosine and S1P in determining cell fate (Cuvillier et al. 1996). According to this concept, the balance of the ceramide, sphingosine and S1P levels defines whether cells undergo proliferation or apoptosis or senescence or differentiation The anti-apoptotic role of S1P has also been linked to the promotion of macroautophagy (simply termed as autophagy, a self-degradation process) (Lavieu et al. 2006). Autophagy maintains cellular homeostasis by regulating turnover of cellular components and plays important roles in determining cell fate (Green and Levine 2014). Autophagy can promote cell survival or cell death depending on which autophagy gene products are switched on (Codogno and Meijer 2005). Emerging evidence suggests that ceramide has a complex role in autophagy, promoting cell death in a context-dependent manner (Jiang and Ogretmen 2014). Ceramide also has diverse cellular targets, such as protein phosphatase 2A, p38, JNK, PKC, survivin and other proteins that regulate cell cycle and apoptosis (Morad and Cabot 2013). On the other hand, sphingosine was initially found to inhibit protein kinase C, exert anti-proliferative effects and exhibit cell-type specific functions (Hannun and Bell 1989). Sphingosine also regulates nuclear lipid signaling as it has been shown to bind the nuclear receptor, Steroidogenic Factor-1, that mediates steroid biosynthesis, and primase that regulates synthesis of RNA primers (Simbulan et al. 1994; Urs et al. 2006). More recently, sphingosine has been found to activate Translesion DNA Polymerase to promote cell proliferation (Kamath-Loeb et al. 2014). Moreover, the cellular effects of sphingosine are

cell-type and cell-context dependent. Notably, sphingosine can be converted to S1P by the action of SK1 or SK2 to stimulate cell proliferation. S1P functions both intracellularly (by binding to its target proteins) and extracellularly (through agonism of cell surface S1P-specific receptors). Moreover, the S1P signalling axis is implicated in many human diseases including inflammation and cancer (Dilshara et al. 2014; Kunkel et al. 2013; Pyne and Pyne 2010), providing opportunities for development of SK1 or SK2 inhibitors and S1P receptor agonists or antagonists as therapeutic agents.

2.3 S1P signaling

S1P acts on extracellular receptors ($S1P_{1-5}$) to regulate diverse cellular responses, such as cell proliferation, migration, differentiation and immune cell trafficking. Each receptor is preferentially coupled to a subset of G proteins that regulate various effectors. The effects of S1P receptor-mediated signaling depend on the relative expression of S1P receptor sub-types. S1P also acts intracellularly to promote epigenetic regulation, calcium mobilization, mitogenesis, inflammation and apoptosis suppression. Hence, to dissect the effects of S1P on cells, both extracellular and intracellular actions have to be considered.

SK1 is activated in response to stimulation of many receptor types. This involves SK1 translocation to the plasma membrane where S1P is formed. S1P may then be transported actively across the lipid bilayer (Kim et al. 2009) to act as an autocrine signal ("inside-out" or sequential signaling (Takabe et al. 2008)). By contrast, integrative signaling can enhance the co-migratory effect of extracellular S1P with other growth factors acting via S1P receptors and receptor tyrosine kinases (Waters et al. 2006).

In the circulation, immune cell trafficking is regulated by a S1P gradient (high S1P concentration in blood and lymph, low S1P concentration in tissues) (Hla et al. 2008). The S1P transporter spinster homolog 2 (Spns2) is responsible for S1P export from lymphatic endothelial cells, which mediates lymphocyte egress (Fukuhara et al. 2012). In the plasma, S1P is carried by apolipoprotein M in a complex with high-density lipoprotein (HDL) (Christoffersen et al. 2011). Apolipoprotein M not only functions as a vehicle to carry and deliver its S1P to different tissues but to also stimulate S1P synthesis in the liver (Liu et al. 2014) and to induce insulin secretion (Kurano et al. 2014).

Therapeutic targeting of S1P signaling has gained momentum with the recent resolution of the crystal structures of SK1 (Wang et al. 2013), $S1P_1$ (Hanson et al. 2012) and S1P lyase (Bourquin et al. 2010). Molecular interactions between inhibitors and SK1 can now be modeled accurately to elucidate the structure-activity relationship of these inhibitors (Baek et al. 2013). The structure also provides an avenue for the study of potential allosteric modulators of SK1 (Lim et al. 2012b; Lim et al. 2001a; Liu et al. 2013). SK2, a larger protein than SK1 and with differing biochemical properties and distribution, is less well characterized. Nevertheless, SK2 appears to play a contributory role in cancer and inflammatory diseases as evidenced by the therapeutic effects observed with SK2 inhibitors *in vitro* and in preclinical disease models (Lim et al. 2011b, Neubauer and Pitson 2013). Therefore, solving the atomic structure of SK2 would accelerate the development of specific SK2 inhibitors and advance our understanding of the role of SK2 in various diseases. Similarly, the crystal structure of $S1P_1$ provides mechanistic insights into how this receptor can be effectively targeted. Interestingly, the N-terminus and extracellular loops of the receptor blocks ligand access; S1P appears to slide horizontally in the transmembrane region to enter the binding pocket (Hanson et al. 2012). Since the only approved $S1P_1$ modulator (FTY720) is not selective, development of specific $S1P_1$ modulators is required to avoid effects on other S1P receptors. Several orally active S1P lyase inhibitors are also in preclinical development, offering new approaches for the treatment of autoimmune diseases like multiple sclerosis and rheumatoid arthritis (Bagdanoff et al. 2010; Weiler et al. 2014).

Targeting S1P production or removal may affect receptor-mediated actions of S1P (i.e. extracellular) but also its intracellular actions. For example, S1P has been found to play a role in the nucleus by binding to and inhibiting histone deacetylases (HDACs) (Hait et al. 2009). Phorbol ester can activate SK2 to increase S1P levels which bind to and inhibit HDAC1/2. This leads to up-regulation of cyclin-dependent kinase (CDK) inhibitor *p21* and the proto-oncogene c-*fos*. This suggests that intracellular S1P is an important epigenetic regulator (Hait et al. 2009). In addition, S1P binds TRAF2 in the cytoplasm and increases TRAF2-dependent polyubiquitination of receptor interacting protein 1 (RIP1). RIP1 is a scaffold protein for IKK-dependent regulation of the NF-κB pathway that regulates pro-inflammatory responses (Alvarez et al. 2010). Intracellular S1P also regulates mitochondrial assembly and respiration by binding to a mitochondrial protein, prohibitin 2 and promoting respiratory complex IV assembly (Strub et al. 2011). In

addition, intracellular S1P can bind to and enhance β-site APP cleaving enzyme-1 (BACE1) activity to increase β-amyloid production, which contributes to the pathogenesis of Alzheimer's disease (Takasugi et al. 2011). More recently, S1P binding to cellular inhibitor of apoptosis 2 (cIAP2) has been shown to trigger interferon-regulatory factor 1 (IRF1) polyubiquitination that is essential for IL1-dependent expression of CXCL10 and CCL5 chemokines (Harikumar et al. 2014). Recently, FTY720 phosphate (the SK2 phosphorylated derivative of FTY720) was shown to inhibit HDACs in a fashion similar to S1P and to eliminate fear memories in mice, implicating a new role of S1P in memory formation (Hait et al. 2014). Together, these studies consolidate our understanding on S1P signaling in normal and pathological processes and reveal new therapeutic opportunities.

3. Effects of resveratrol on sphingolipids in disease

The prevalence of cancer, cardiovascular, neurodegenerative diseases and metabolic disorders increases with age. In this regard, the effects of resveratrol in slowing the progression of age-related diseases are well documented (Baur et al. 2012). Interestingly, sphingolipids, such as ceramide and dihydroceramide have been shown to modulate lifespan of yeast via autophagy and activation of AMPK signaling that is independent of sirtuin activation (Huang et al. 2014). Since resveratrol modulates sphingolipid levels, the beneficial effects of resveratrol such as anti-cancer, anti-inflammatory activities, cardio- and neuro-protective properties might be tightly linked with changes in sphingolipid signaling. The following section provides evidence for the role of sphingolipids in mediating the activities of resveratrol in multiple human diseases.

3.1 Cancer and Inflammation

Resveratrol alters sphingolipid levels through multiple mechanisms. Firstly, resveratrol increases ceramide to induce apoptosis in colon, breast and prostate cancer cells. Similarly, synthetic resveratrol analogues with minor side-chain modifications induce ceramide-dependent apoptosis (Minutolo et al. 2005). The effect of resveratrol is through increased *de novo* ceramide biosynthesis and is reversed by SPT inhibitors (Dolfini et al. 2007; Sala et al. 2003; Scarlatti et al. 2007; Scarlatti et al. 2003; Ulrich et al. 2007). However, ceramide levels increase 24-48 hours after resveratrol treatment (Scarlatti et al. 2003), which correlates

with the onset of apoptosis (Delmas et al. 2003; Dimanche-Boitrel et al. 2005), and suggesting *de novo* changes in the expression of enzymes involved in ceramide metabolism. The mechanisms by which resveratrol and ceramide induce apoptosis in ovarian cancer cells are partially overlapped and involve a COX-2-dependent pathway (Lin et al. 2013). Resveratrol also increases dihydroceramide levels by reducing the activity of dihydroceramide synthases in gastric cancer cells (Shin et al. 2012). There is also increasing evidence that resveratrol can induce autophagy by modulating sphingolipid levels in cancer cells. In this regard, resveratrol increases dihydroceramide levels and induces autophagy that is possibly linked with inhibition of growth of HCG-27 gastric cancer cells (Signorelli et al. 2009). In addition, resveratrol has been shown to induce autophagic cell death in human breast cancer (Scarlatti et al. 2008), colorectal cancer (Trincheri et al. 2008), ovarian cancer (Kueck et al. 2007) and chronic myelogenous leukemia cells (Puissant et al. 2010). Therefore, resveratrol induced changes in ceramide, dihydroceramide and S1P levels appear linked with inhibition of proliferation, stimulation of autophagy and induction of cancer cell death.

Blockade of NF-κB signaling is one of the early events observed with resveratrol treatment. For example, resveratrol abrogates TNF-induced activation of NF-κB within 30 minutes of treatment (Ashikawa et al. 2002; Manna et al. 2000). Moreover, pre-treatment of endothelial cells with SGE (a red grape skin polyphenolic extract, containing resveratrol oligomers) reduces the responsiveness of these cells to S1P-induced migration, ERK1/2 activation and platelet-activating factor synthesis (Barthomeuf et al. 2006). NF-κB is a proposed downstream effector of S1P (Alvarez et al. 2010). Therefore, the effects of SGE in blocking S1P-dependent angiogenesis might lie upstream of NF-κB . Indeed, Issuree and colleagues (2009) showed that resveratrol inhibits SK1-induced inflammatory response triggered by C5a (a chemoattractant cytokine). Resveratrol also inhibits activation of phospholipase D (PLD) which is an upstream activator of SK1 (Issuree et al. 2009). Dietary polyphenols including resveratrol also down-regulate SK1 expression and activity in prostate cancer cells (Brizuela et al. 2010). These studies suggest that resveratrol inhibits the ERK/PLD pathway which is upstream of SK1 activation. However, direct inhibition of purified SK1 activity by resveratrol has been observed and treatment of MCF-7 breast cancer cells with resveratrol induces down-regulation of SK1 expression (Lim et al. 2012a). The inhibition of SK1 activity by resveratrol is likely to disrupt the sphingolipid rheostat and to increase ceramide levels. This might account for the ability of resveratrol to induce apoptosis of MCF-7 breast cancer cells (Lim et al. 2012a). Moreover,

balanocarpol and its isomer, ampelopsin A also inhibit SK1 activity and down-regulate SK1 expression, which induces apoptosis in MCF-7 breast cancer cells (Lim et al. 2012a). Interestingly, resveratrol up-regulates ceramide synthases and down-regulates SK1 expression in myeloid leukemia cells (Cakir et al. 2011). Resveratrol also reduces activity of SK1 in a rat model of ulcerative colitis and ablates severity of oxazolone-induced inflammation (Abdin 2013). Perturbation of the sphingolipid rheostat by resveratrol and oligomers might therefore be partly responsible for the anti-inflammatory and anti-cancer activities of these compounds.

3.2 Cardiovascular disease

Circulating S1P is bound to HDL (~ 55%), albumin (~ 35%) and LDL/VLDL (< 10%) and is derived from vascular endothelial and red blood cells in a range between 200-900 nM (Poti et al. 2014). At this concentration, S1P dissociates from its carrier proteins to exert cardiovascular and atheroprotective effects by binding to S1P receptors. Indeed, circulating S1P or activation of intracellular SK1 confers protection against myocardial infarction and acute ischemia/reperfusion injury (Karliner 2013). Even though the overall effect of S1P on the heart and vasculature appears protective after injury, several studies report conflicting results due to the pro-fibrotic, heart muscle thickening and vasoconstrictive properties of S1P (Waeber and Walther 2014). For instance, $S1P_3$ is linked to bradycardia, an undesirable adverse effect of FTY720 phosphate (Means and Brown 2009). Therefore, compounds selectively targeting the $S1P_1$ receptor might be better suited for the treatment of myocardial infarction (Waeber and Walther 2014). The consensus is that further investigations employing larger animal models, selective pharmacological tools and more epidemiological studies are required to improve our understanding of the role of S1P and thereby enable development of new therapeutic agents for treatment of cardiovascular disease.

Despite favorable outcomes observed for resveratrol and other polyphenolic compounds in many *in vitro* and *in vivo* models of cardiovascular disease, the evidence for cardioprotection is not unequivocal in humans based on clinical and epidemiological evidence (reviewed by (Khurana et al. 2013; Tome-Carneiro et al. 2013). For example, even though resveratrol appears beneficial against myocardial infarction in animal models, several recent large-scale analyses have argued against a protective effect of resveratrol in humans. In addition, the effect of resveratrol on lipoprotein metabolism is not significant in a meta-analysis

of seven randomized controlled trials (Sahebkar 2013). Similarly, Semba and colleagues did not find an association between resveratrol and any health benefits after following 783 individuals for 9 years (Semba et al. 2014). More long-term controlled trials will assess the use of resveratrol as a dietary supplement for prevention or as a drug for treatment of cardiovascular diseases.

Since resveratrol and its oligomers modulate sphingolipid metabolism and increases pro-apoptotic ceramide levels, future studies should explore whether this effect is cell-type specific. For example, due to constant exposure to UV irradiation, keratinocytes have evolved a protective mechanism to convert ceramide to S1P by activating ceramidase and SK1 (Uchida 2014). Intriguingly, resveratrol increases ceramide levels (via activation of SPT) and S1P formation (by increasing SK1 mRNA expression), which leads to increased antimicrobial defense in human skin cells (Park et al. 2013). Therefore, possibly through this metabolic conversion of ceramide to S1P, resveratrol may have a protective role in some cell types. Low concentration of resveratrol (< 50μM) increases sphingosine, ceramide and S1P levels in human skin cells but high concentration of resveratrol reduces cell viability (Park et al. 2013). This is consistent with the effect of resveratrol in cancer cells where high concentration (> 50μM) of resveratrol induce apoptosis possibly involving inhibition of SK1 activity and down-regulation of SK1 expression (Lim et al. 2012a). Thus, the effects of resveratrol on SK activity and sphingolipid metabolism appear concentration dependent and this will define the effect of resveratrol on cellular outcome.

Therefore, the use of resveratrol to modulate sphingolipid levels and signaling in cardiovascular disease requires careful consideration. Clearly, the ability of resveratrol to increase ceramide levels either by activating SPT or by inhibiting SK1 activity might dictate a pro-apoptotic effect which would be deleterious. Second, the multifaceted roles of S1P in the cardiovascular system make it difficult to harness the beneficial effects of reducing S1P levels while avoiding unwanted side-effects. For example, inhibiting SK1 activity with resveratrol might reduce cardioprotection afforded by S1P associated with HDL and S1P-dependent activation of pro-survival signaling such as PI3K/Akt and MAPK pathways. However, S1P can reduce heart rate ($S1P_3$) and constrict coronary arteries ($S1P_2$) (Waeber and Walther 2014) and these effects would be potentially alleviated by inhibiting SK1 activity/expression with resveratrol. Indeed, resveratrol has been shown to be effective in ameliorating hypertension, ischemic heart disease and

cardiomyopathy in animal studies (Raj et al. 2014). A recent study has shown that resveratrol is effective in reducing arterial wall inflammation and stiffening (hallmarks of vascular aging) in rhesus monkeys fed with high fat diet, possibly through activation of the Nrf2 anti-oxidant pathway (Mattison et al. 2014). Resveratrol also exhibits anti-oxidant and anti-inflammatory properties due to modulation of other signaling pathways that might impact more significantly on cardioprotection than the modulation of S1P signaling. These include activation of AMPK, SIRT1 and NO signaling as well as inhibition of pro-inflammatory cytokine signaling such as the NF-κB pathway (Raj et al. 2014). Therefore, further investigations are needed to examine the effect of resveratrol on sphingolipid levels in cardiovascular disease models. Investigation of the activity of resveratrol oligomers which might be "fine-tuned" in specificity of action is also worthwhile, particularly given their ability to inhibit SK1 activity. Long-term studies are also needed to assess the side effects and toxicity of these compounds.

3.3 Neurodegenerative disease

Altered sphingolipids metabolism is linked to multiple neurodegenerative diseases, such as Alzheimer's disease, Niemann Pick disease, amyotrophic lateral sclerosis, Parkinson and AIDS dementia (Mielke and Lyketsos 2010). The strongest evidence that modulation of sphingolipids by resveratrol might impact disease progression is in Alzheimer's disease. Alzheimer's disease is known to affect the elderly and is responsible for memory loss as a result of dementia. Central to the pathogenesis of Alzheimer's disease is BACE1 (also referred to as β-secretase), which is an enzyme responsible for the formation of neurototoxic β-amyloid and therefore, is a therapeutic target (Yan and Vassar 2014). S1P produced by SK2 has been found to be neurotoxic in S1P-lyase deficient mice (Hagen et al. 2011). SK2, located at the ER induces apoptosis by generating intracellular S1P or phosphorylating sphingoid-based substrates (Don et al. 2007; Maceyka et al. 2005). Moreover, knockdown of SK2 by siRNA or inhibition of SK2 activity with ABC294640 (a specific SK2 inhibitor, (French et al. 2010)) reduces β-amyloid production in N2a neuroblastoma cells, indicating that SK2-derived S1P activates BACE1 to increase β-amyloid level in mouse neurons (Takasugi et al. 2011). Therefore, inhibition of SK2 activity is predicted to have therapeutic value in reducing formation of β-amyloid. Inhibition of SK1 activity by resveratrol is likely to be counteracted by the fact that ceramide (which is increased by resveratrol) which might accumulate also

increases β-amyloid levels (by increasing BACE1 stability) (Puglielli et al. 2003) and induces oxidative stress in neurons (Cutler et al. 2004). Interestingly, other lipids (e.g. neutral glycosphingolipids, anionic glycerolipids and sterols) have also been shown to stimulate BACE1 activity in a reconstituted proteoliposome system (Kalvodova et al. 2005). Moreover, an increased ceramide/S1P ratio favors apoptosis that might result in further neurodegeneration. Indeed, ceramide levels are high in Alzheimer's patient brain region that exhibit β-amyloid accumulation (Cutler et al. 2004). In addition, serum ceramide is also associated with increased risk of developing Alzheimer's disease in woman (Mielke et al. 2012). Moreover, down-regulation of SK1 or up-regulation of S1P lyase (both of which would likely result in reduced S1P level) expression are found in Alzheimer's patient brain tissue, suggesting that high ceramide/S1P ratio in the brain is neurotoxic (Ceccom et al. 2014; Couttas et al. 2014). Therefore, SK inhibitors (e.g. 2-(p-hydroxyanilino)-4-(p-chlorophenyl)thiazole, abbreviated as SKi) that inhibit both SK1 and SK2 activity would appear counterproductive (Lim et al. 2011b; Loveridge et al. 2010; Mielke and Lyketsos 2010). In addition, SKi has been found to increase dihydroceramide levels, possibly by inhibiting dihydroceramide desaturase (Cingolani et al. 2014; Loveridge et al. 2010). Dihydroceramide might also be linked to autophagy and ER stress, which mediates G0/G1 cell cycle arrest and suppresses cell proliferation (Gagliostro et al. 2012). In addition, defects in autophagy-related pathways are implicated in neurodegeneration (Nixon 2013). Substantial evidence has now demonstrated that sphingolipids regulate both autophagy and apoptotic pathways to determine cell survival (Li et al. 2014).

In Alzheimer's disease, resveratrol ameliorates various pathological features associated with β-amyloid and Tau protein misfolding, inflammation, cell metabolism, telomere shortening, mitochondria dysfunction and oxidative stress (Granzotto and Zatta 2014; Vingtdeux et al. 2008). A beneficial effect has been demonstrated lately for resveratrol supplementation in elderly patients who exhibit better memory performance associated with improved glucose metabolism and hippocampal functional connectivity (Izeddin 2014). Other polyphenols have been shown to penetrate the brain and exhibit anti-amyloidogenic activity that improves cognitive function in a mouse model of Alzheimer's disease (Wang et al. 2014). Indeed, it would be interesting to investigate the effect of resveratrol oligomers found in the red grape skin polyphenolic extract, SGE (Baechler et al. 2014). Thus far, several resveratrol oligomers are known to inhibit cholinesterase (Table 1) and suppress β-amyloid formation (Richard et al. 2011a), which might

therefore benefit Alzheimer's patients. For example, ε-viniferin glucoside (a resveratrol dimer), has been shown to reduce β-amyloid aggregation and toxicity *in vitro* (Richard et al. 2011b). No formal assessment has been made in relation to changes in sphingolipid levels in neurons after treatment with resveratrol. Nevertheless, neuroprotection has been consistently observed at < 50μM of resveratrol, which is lower than the concentration required to inhibit SK1 activity (K_i=160 ± 40μM) and to induce apoptosis (Capiralla et al. 2012; Chen et al. 2005; Feng et al. 2013; Lim et al. 2012a; Marambaud et al. 2005; Wang et al. 2014). Therefore, low micromolar concentrations of resveratrol might mediate protection via a mechanism that does not involve changes in sphingolipid levels. A recent study has demonstrated that the resveratrol sulfate metabolites could effectively be taken up by cells and converted back to resveratrol *in vivo* (Patel et al. 2013). Thus, caution is needed because resveratrol might be actively taken up by neurons, which could result in higher intracellular than plasma concentration.

3.4 Metabolic disease

Ceramide has been implicated in the pathogenesis of metabolic diseases; for instance, by promoting insulin resistance, lipotoxicity and cell death (Russo et al. 2013). Significant changes in sphingolipid levels and up-regulation of ceramide-producing enzymes have been observed in obese rodents, which are accompanied by activation of pro-inflammatory cytokine signaling (Samad et al. 2006). Indeed, pro-inflammatory receptor TLR4-mediated signaling leads to the activation of NF-κB and up-regulation of genes involved in the generation of ceramide and insulin resistance. In addition, ceramide inhibits pro-survival Akt signaling to induce cell death and suppress insulin sensitivity (Holland et al. 2011a; Maceyka et al. 2012). By contrast, pro-survival S1P stimulated by adiponectin (a hormone with anti-inflammatory function) activates AMPK (in a S1P receptor-dependent manner), which is required for mitochondrial biogenesis (Holland et al. 2011b; Maceyka et al. 2012). Therefore, reducing the ceramide/S1P ratio is an attractive therapeutic goal for the management of diabetes.

Resveratrol has been shown to improve insulin sensitivity and protect against metabolic disease in mice fed with high-fat diet. This is associated with activation of AMPK/SIRT1/PGC-1α pathway (Baur et al. 2006). Moreover, resveratrol might exhibit anti-obesity action by reducing the body weight and abdominal fat of experimental mice (Baek et al. 2014). However, translation of these beneficial effects to the clinic has been

difficult. For example, there is a lack of physiological responses and no gene expression changes of metabolic and inflammatory biomarkers in obese man taking a high dose of resveratrol. Indeed, a recent review on all available clinical trials or interventions of obesity and Type II diabetes has found no significant benefits associated with resveratrol consumption (Timmers et al. 2013). Of note, clinical trials measuring health benefits might be difficult to design, which requires careful selection of patients, considering their health status, underlying morbidity, age, gender and sex. In addition, improved health and lifespan extension has been observed only in mice having high-fat diet but not in lean mice, suggesting that resveratrol might have a beneficial effect on patients with severe metabolic derangement. More controlled clinical studies are needed to investigate the therapeutic potential of resveratrol in metabolic diseases and its effects on sphingolipid metabolism.

4. Summary and future direction

The role of sphingolipids in the pathogenesis of human disease is widely recognized. During the past few years, more information regarding the therapeutic potential of resveratrol has also accumulated. Importantly, resveratrol can oligomerize into larger molecules with better potency than resveratrol and in some cases, better selectivity. More work is needed to investigate the *in vivo* activity of resveratrol oligomers and to assess their therapeutic efficacy and toxicity. We consider that changes in sphingolipid metabolism in response to resveratrol and related oligomers are likely to be beneficial in the treatment of cancer patients and, more speculatively, in cardiovascular disease patients. However, changes in sphingolipid metabolism in response to resveratrol in metabolic disorders and neurodegenerative diseases are contraindicated and likely deleterious. This conclusion is based on findings that the effects of resveratrol on sphingolipid metabolism and disease progression are concentration-dependent. At low micromolar concentrations of resveratrol, which exhibit cardioprotective and neuroprotective properties, there is minimal perturbation of sphingolipid metabolism (excluding cellular uptake and increased local concentration). However, at high concentration (> 50μM), resveratrol increases pro-apoptotic ceramide and reduces pro-survival S1P levels, which suppresses cell growth and induces apoptosis. Therefore, a better understanding of the long-term effects of resveratrol (and its oligomers) on S1P- and ceramide-dependent

modulation of disease progression is required. Nevertheless, the potential of the resveratrol/sphingolipid partnership might offer new avenues for the treatment of cancer.

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