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Exploring Concentration and Duration Dependence in Hormesis

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ABSTRACT

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The phenomenon of hormesis, characterized by beneficial low-exposure stress-protective effects, has experienced increasing interest by the scientific community. Elucidating the underlying cellular signaling pathways that lead to a new homeostatic state can provide crucial information about new therapeutic targets to prevent and treat diseases. Commonly investigated for hormetic behavior are plant-based substances (phytochemicals), such as quercetin, epigallocatechin gallate, curcumin, and resveratrol. The polyphenol resveratrol, found in peanuts, grapes, and subsequently in wine, is known for a variety of effects - beneficial and detrimental - depending on the investigated cell type and concentration. In HepG2 cells, an increase in proliferation has been observed previously at doses between 10 µM to 100 µM after 16 hours, while higher doses decreased cell viability markedly, eventually leading to cell death. For other compounds, literature searches provide conflicting data on the hormetic capabilities and subsequently on the associated concentrations. This work aims to clarify the concentration and exposure duration dependence in hormesis. The four aforementioned compounds were screened for their hormetic behavior in a human liver cancer model by assessing the effects of varying concentrations on metabolic activity and plasma membrane integrity. Quercetin, epigallocatechin gallate, and curcumin did not show hormetic properties in the investigated concentration range during a 24-hour exposure. Resveratrol qualified for further experiments where cells were subjected to 0.1 to 500 µM for six, twelve, 24, 48, and 72 hours. A significant change in metabolic activity at concentrations of 50, 100, and 250 µM was found between twelve and 24 hours, as well as a significant increase in plasma membrane degradation after 24 hours at doses higher than 100 µM. This demonstrates a noticeable change in cellular behavior between the twelve- and 24-hour mark, associated with resveratrol concentrations from 50 to 250 µM. Future research should include real-time assessment of energy markers, preferably up to 24 hours, to pinpoint timepoints of metabolic switches, and subsequently study the changes that underlie hormesis at these timepoints.

DEDICATION

This thesis is dedicated to Katharina Brylla, an extraordinary women, mother, and grandmother. She persisted through unimaginable adversities in the darkest times of European history without losing herself. Not everyone can live a happy and productive life after these experiences and enjoy the small things in life, including advocaat and “Mensch Ärgere Dich Nicht”. She was the inspiration for long days and short nights in the hope of spending a few more joyful moments in her presence after graduation. Unfortunately, I was too late.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
AMPK	adenosine monophosphate-activated protein kinase
Atg	autophagy-related
ATP	adenosine triphosphate
CDR	cell danger response
DISC	death-inducing signaling complex
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulfoxide
EDTA	ethylenediaminetetraacetic acid
ETC	electron transport chain
FAD^+	flavin adenine dinucleotide
$FADH_2$	reduced flavin adenine dinucleotide
FBS	fetal bovine serum
GCK	glucokinase
GCN2	general control nonderepressible 2 protein
GLUT2	glucose transporter 2
GPCR	G-protein coupled receptor
hOGG1	8-oxyguanine DNA glycosylate
MT	metallothioneins
mTOR	mammalian/mechanistic target of rapamycin
mTORC1	mammalian/mechanistic target of rapamycin complex 1
MTT	3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
NAD^+	nicotinamide adeninde dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adeninde dinucleotide phosphate

NADPH	reduced nicotinamide adenine dinucleotide phosphate
Nrf2	nuclear factor (erythroid-derived 2)-like 2
PBS	phosphate buffered saline
PCD	programmed cell death
RAPTOR	regulatory-associated protein of mTOR
ROS	reactive oxygen species
SIRT1	NAD-dependent deacetylase sirtuin-1
TNF	tumor necrosis factor
tRNA	transfer RNA (ribonucleic acid)
TSC2	tuberous sclerosis complex 2
ULK1	UNC-51 like kinase 1

1. Introduction: Aspects of Cellular Signaling, Energy Demand, and Hormesis

Multicellular organisms are in a constant fight of survival with the environment and everything stands in exchange of matter and energy. It is a battle on two fronts: the physical environment, which includes insults from sunlight, dryness, wind, storm, or cold, and the chemical environment, represented by myriads of harmful and/or beneficial micro- and macromolecules.[1,2] Every living species on this planet, at any given moment in history up until now, has evolved and adapted to the direct environment, in an effort to maximize the likelihood of survival and procreation. Adaptions can be long lasting changes in the genetic sequence, or in its expression, that affect subsequent generations, but often are transient changes to handle immediate exposures. On the molecular and cellular level, this can be achieved by settling for a state of equilibrium, that is different than the state before, particularly in hormesis.

The phenomenon of hormesis is commonly described as a biphasic dose-response behavior, where low doses of a compound exert a beneficial effect on cells or tissue, while higher doses prove detrimental.[3–6] More broadly, it can also be stated with the familiar saying: "what does not kill you, makes you stronger". Rigorous exercise and simultaneous energy restriction have long been seen as the original trigger for hormesis, based on a switch between "stress resistance mode" and "growth/plasticity mode"[1]. Short periods of ischemia also appear to "precondition" the heart for a more severe ischemic event.[7] However, the principle of hormesis has already been described in the late 19th century by Prof. Hugo Schulz, when he observed stimulation of bacterial metabolism through bactericidal agents at low doses.[8] The underlying concept of small amounts of injury strengthening the system in the long run has been attributed to the effects on receptor signaling, signaling pathways such as the mammalian (mechanistic) Target of Rapamycin, and cellular antioxidant systems.[4,8,9] Theories on how constant low-level stress can be beneficial include two aspects: an increase in repair behavior, and an adaption towards a more stress-resistant state within the cell. In the early years of hormesis research, studies focused on toxic substances, including metals, but the scope of a "mild stressor" has been broadened to include environmental stimuli as well, especially plant-based substances.[4,7] Generally, a hormetic effect is attributed to a stressor, may it be of physical, nutritional, or environmental origin, when the dose-response assessment has

biphasic character (inverted U- or J-/U- shaped curves).[10,11] These curves display a hormetic zone, where the associated dose stimulates the assay response up to 30 - 60% of the control (significantly, especially with respect to the physiological consequences).[8,10] Figure 1 shows exemplary curves if one were to measure cell viability and cell death in a dose-dependent manner: The hormetic zone is then defined as the area where a decrease in cell death to a percentage less than the control value or an increase in cell viability above control values is observed.

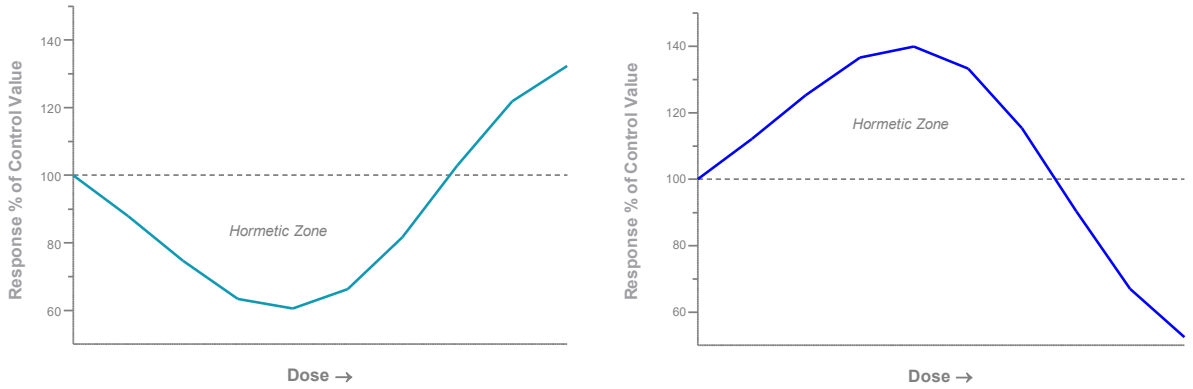


Figure 1| Stylized curves illustrating a dose-response curve that shows hormetic behavior.

On the left, a U-shaped curve as it would ideally be found when measuring cell death, for example. On the right, an inverted U-shaped curve, as it would ideally be found when measuring cell viability, for example.

To assess, understand, take advantage of, or undo a compound's effect on a system, it is necessary to characterize the cellular signaling associated with the molecule. While it is important to know how the molecule distributes throughout the system, e. g. where it is absorbed, stored, or metabolized, it is even more important to detect the changes the molecule and its metabolites evoke in the systems smallest unit, the cell, and the associated time course.[12]

1.1. Cellular Signaling: Function and Cellular Constituents Involved

A xenobiotic insult generates a variety of responses inside a cell, a whole machinery of different mechanisms that deal with the foreign substance and its metabolites. A lot of these mechanisms are evolutionary conserved and the entirety of the responses are termed the cell danger response (CDR).[2] For the machinery to deal with the xenobiotic successfully, all incorporated pathways

need to communicate with each other across cellular compartments and membranes in signal transduction.

1.1.1. Membranes

Lipophilic in nature, the cell membrane core establishes a very effective barrier between the extracellular environment and the cytosol as well as between the cytosol and organelle matrices. Only select molecules can diffuse freely through the semipermeable membranes, based on their size and hydrophobicity. Passive diffusion through the membrane is possible for uncharged, lipophilic molecules that "dissolve" in the membrane (e.g. steroid hormones derived from cholesterol).[13] Polar molecules and ions, except water, cannot freely cross the membrane due to their lipophobic character. Many chemical species, such as metabolic products from carbohydrate and protein metabolism, as well as electrolytes, and growth factors, must transfer in and out of cells. Not every molecule needs to enter the cell, to communicate the environmental status quo. Eukaryotic cells are well-equipped to sense signals outside of the cell, amplify them inside the cell, and initiate an appropriate response. Membrane spanning sensor proteins, or receptor proteins, which are present on cell and organelle membranes, are capable of binding a ligand on one side (e.g. extracellular) and activating secondary messengers on the other side (e.g. in the cytosol).[14] They thereby propagate the signal into the cell. In return, the cell can signal its status quo to the surrounding environment through lipid vesicles and exosomes, for example.

1.1.2. Signaling in the Mitochondria

The mitochondria has been recognized as a highly important organelle in cell signaling as it is actively involved in apoptosis, autophagy, immune responses, and calcium signaling.[15] These functions are intimately interwoven: cell death in the form of apoptosis is considered the first-line of defense against incoming pathogenic threats. At the same time, cell death initiates an immune response to clear the pathogenic threat, which can be controlled through autophagy as a pro-survival mechanism.[15] The mitochondria is, at the same time, considered the "powerhouse" of the cell, due to its role in energy production in the form of adenosine triphosphate (ATP).

As a side-effect of energy production in the mitochondria, reactive oxygen species (ROS) are produced due to electrons leaking from the electron transport chain. Despite the overall harmful effects of ROS on cellular molecules, recent research suggests a beneficial impact from low concentrations of ROS. It is thought that these concentrations may stress the cell just enough to cause adaption and prolong longevity instead of cell death,[16] which might even decrease the pace of aging. Ji *et al.* (2010), Sauer *et al.* (2001), and Linley *et al.* (2012) connect an increase in hydrogen peroxide from immediate ROS detoxification, and ROS in general, to the enhanced regulation of embryonic stem cell differentiation, intracellular cell growth, and neurokinin signaling in sensory neurons, respectively.[17–19]

1.1.3. Temporal and Spatial Aspects of Signaling

A cell is prepared to respond to signals in various places, and the duration and ramification of the signal vary significantly based on the nature of the stimulant. The duration of a signal depends on the pathways involved, and cellular compartmentalization offers distinct locations for distinct functions. Reaction kinetics and substrate availability also dictate the temporal aspect of signaling, since some reactions take longer than others.[12,20] Not every process occurs everywhere in the cell; organelles have a specific microenvironment that increases the likelihood of certain reactions more over others. The early pictures of linear signal pathways made way for complex signaling cascades and networks, that can be highly conserved, such as signaling through cell cycle checkpoints, or can be highly complex and even vary for the same signal molecule depending on the tissue type.

The basis of spatially distributed signals and reactions is a difference in diffusivity for active/inactive forms of signaling molecules. While it is known that the activity of an enzyme changes with posttranslational modification, the diffusivity also changes, creating separate concentration gradients for the two forms of the same molecule. Additionally, due to slightly changed physical properties, the preference in microenvironment can change, and therefore the localization within the cell.[21] For example, when a ligand binds to a G-protein coupled receptor (GPCR) on the membrane surface, a kinase causes a secondary response right inside the membrane. Through the secondary response molecule, another kinase more distant from the membrane is activated, and so on and so forth. Signals are eventually terminated by phosphatases in the cytosol, but not without branching into and initiating more signaling.[20] The exact location

of termination, closer or not so close to the membrane, depends on the strength (concentration) of the original GPCR signal. The stronger the signal, the longer the transmission range inside the cytosol and the more diverse pathways can theoretically be activated. This also ties into the temporal aspect of signaling, since it will not take as long to activate a pathway close to the membrane as it does when it is initiated somewhere in the cytosol, spatially further away from the original signal. Another example is the initiation of apoptosis, where several pathways at several locations (receptors, mitochondria, endoplasmic reticulum, etc.) can initiate a cascade of signals that eventually seem to converge at the mitochondria and then start the cleavage of procaspases inside the cytosol.[22]

1.2. Cellular Energy Requirement, Storage, and Metabolism

Cellular signaling is especially important to communicate the metabolic state of a cell within the cell and to the environment. Intermediates in metabolic pathways are therefore also considered signaling molecules and can import information. Metabolic processes are either of anabolic nature, to build larger biomolecules from smaller parts, or of catabolic nature, to break down larger biomolecules into smaller parts.[23,24] Catabolic pathways release energy during their breakdown reactions, while anabolic pathways require energy input in the biosynthesis of macromolecules. The energy that was released or needed in these pathways is stored in highly energetic covalent bonds such as phosphate esters and phosphoanhydride bonds found in ATP or nicotinamide adenine dinucleotide phosphate (NADPH).[23,25–27] Coupling of anabolic and catabolic processes connects energy-requiring and energy-releasing reactions, which creates interrelated networks that overall yield an increase in entropy.

In eukaryotic cells, several catabolic pathways cross, interact, feed into each other, and can eventually all lead to the production of energetic resources (Figure 2).[26] Detailed descriptions of the metabolism of the three main macronutrients (carbohydrates, proteins, and lipids) can be found in literature.[23,25,27] Briefly, lipids are oxidized in a step-wise fashion via β-oxidation, yielding acetyl-CoA, NADH and flavin adenine dinucleotide ($FADH_2$), while carbohydrates – once broken down into single glucose molecules – are metabolized in several pathways depending on the availability of oxygen. The first part of glycolysis is universal, oxygen independent, and produces two equivalents of pyruvate, ATP, and NADH. Anaerobic conditions lead to the fermentation of pyruvate into lactate (oxidative process). Aerobic conditions lead to three consecutive pathways

in the mitochondria. First, pyruvate is oxidized into acetyl-CoA while creating an equivalent of NADH, then acetyl-CoA feeds into the citric acid cycle that yields three molecules of NADH and one molecule of $FADH_2$ per acetyl-CoA. Lastly, the energetic coenzymes participate in oxidative phosphorylation in the electron transport chain to produce ATP directly. Proteins are primarily broken down into their respective single amino acids, which are subsequently used for the synthesis of peptides, but in times of energy demand they can feed into cellular respiration and therefore can also serve as an energy resource.[24]

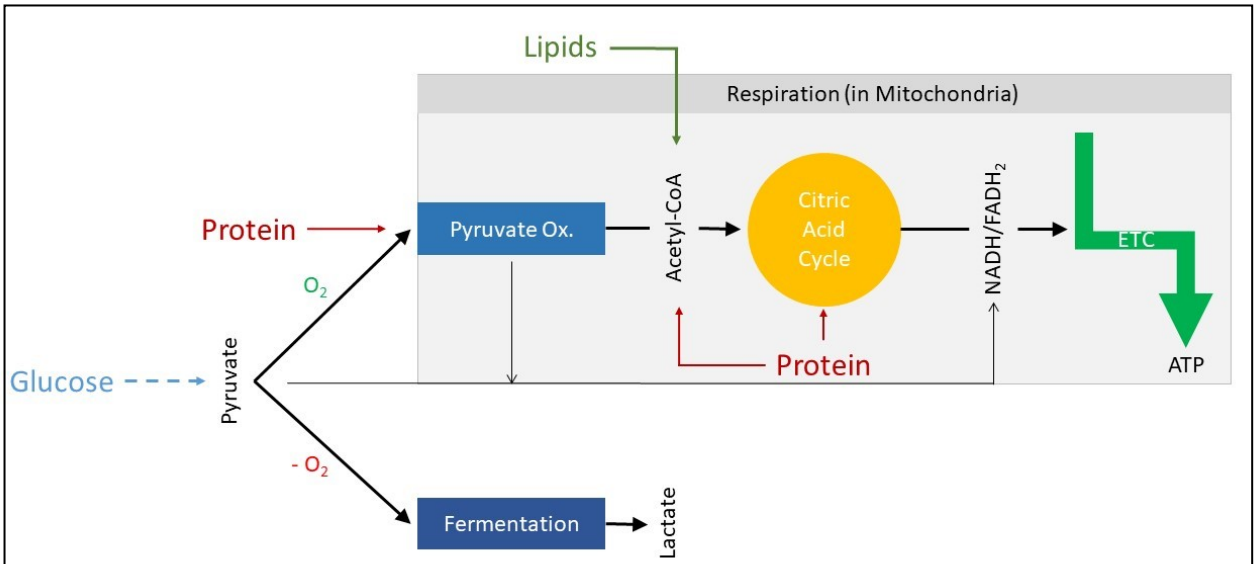


Figure 2| Overview of the converging pathways in carbohydrate, peptide, and lipid metabolism.

1.2.1. Redox Coenzymes: Nicotinamides and Flavins

Oxidation and reduction reactions are often catalyzed by enzymes that depend on redox coenzymes,[28] which transiently store released energy, mostly in the form of one or two electrons.[29,30] Two coenzymes families are important regarding energy metabolism: NAD^+ (nicotinamide adenine dinucleotide) and flavins. The former is the precursor for $NADP^+$ (nicotinamide adenine dinucleotide phosphate).[31] Both are highly involved in cellular metabolism as hydride-transfer enzymes, since they can take up two electrons and a proton to form their reduced counterparts NADH and NADPH.[31] The dephosphorylated form is of special interest in this regard because it can efficiently cause the translocation of a proton at the inner mitochondrial

membrane to establish the proton gradient that is the driving force for ATP production in oxidative phosphorylation. Flavin adenine dinucleotide (FAD) on the other hand is capable of one and two electron transitions, either sequentially or simultaneously.[23] In the electron transport chain (ETC), unpaired electrons from oxidized metabolites can be transferred to diatomic oxygen through $FADH_2$ (Figure 3).[27]

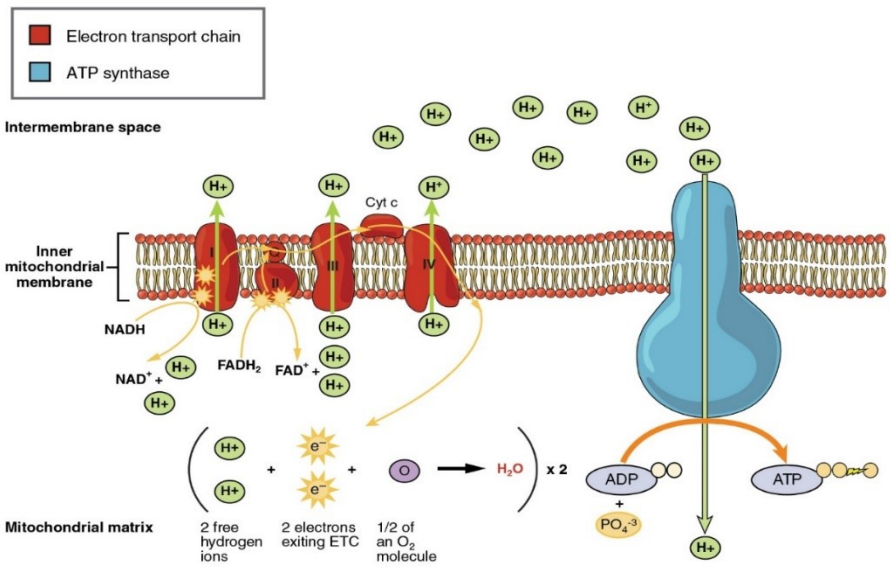


Figure 3| Overview of the electron transport chain and ATP synthase.

The schematic depicts the membrane-bound complexes I through IV and ATP synthase and their action in oxidative phosphorylation: Complex I, III, and IV establish a proton gradient, while Complex II facilitates an electron exchange that ultimately leads to the formation of water. ATP synthase phosphorylates ADP through the motive force from the proton gradient.

Figure reprinted from: Figure 5, OpenStax. *Carbohydrate Metabolism* (OpenStax CNX, 2015). Download for free at http://cnx.org/contents/9d68abf9-4c2e-4ef7-88d1-c963c5c844b9@4.

1.2.2. ATP

ATP is considered the energy currency of the biological metabolism.[32] As Voet and Voet (2011) state it:

> *"the cellular role of ATP is that of a free energy transmitter rather than a free energy reservoir."* [23]

The key to its function lies in the three phosphate groups and the two covalent bonds between them, that hold high potential energy. The first phosphate group attached to the nucleoside is bound through a phosphate ester bond to the 5' oxygen on the nucleoside's ribose. The additional two phosphate groups then form phosphoanhydride bonds to each other (α,β – and β,γ – linkages).[27] The hydrolysis of one mol of ATP to adenosine diphosphate (ADP) has been calculated to release 7.3 kcal/mol under standard condition and 14 kcal/mol under cellular conditions (30.5 kJ/mol and 57 kJ/mol, respectively).[23–25,27] Eukaryotic ATP is directly produced via two pathways: Firstly, through oxidative phosphorylation in the mitochondria, where the membrane proteins of the electron transport chain use the energy from electron carriers to establish a proton gradient in the mitochondrial intermembrane space and couple protons to oxygen to form water (Figure 3). The relief of the proton gradient through the peptide ATP synthase yields the sought-after energy-rich nucleotide. The second pathways is glycolysis in the cytosol, which also releases ATP, but in smaller quantities (two molecules per glucose).[33] Other catabolic pathways indirectly lead to the production of energetic adenosine as well, either by providing glucose, intermediates for the citric acid cycle, or reduced electron carriers. The breakdown of ATP, to adenosine diphosphate and adenosine monophosphate, fuels cellular processes that would otherwise be energetically unfavorable. Additionally, some sources see ATP, ADP, and AMP as a stimulated extracellular signaling molecule and neurotransmitter in purinergic signaling associated with the cell danger response, especially in neuronal cells. Increased secreted ATP from stressed cells then inevitably evokes an immune response.[2]

1.2.3. Metabolic Reprogramming in Cancer Cells

While most cells rely heavily on mitochondrial oxidative phosphorylation to produce ATP, it was observed by Otto Warburg *et al.* at the beginning of the past century, that carcinoma cells turn over glucose into lactose 125x more than blood, up to 200x more than an inactive frog leg muscle, and 8x more than an active frog leg muscle at its maximum capacity under anaerobic conditions; this trend does not change significantly (as it should) under aerobic conditions.[34] The overall findings were, that cancer cells show a higher glycolytic flux via fermentation, even if oxygen is available. This has since been termed the "Warburg Effect".[35] The exact benefits of the altered metabolism for a cancer cell are not fully understood yet,[5] but several theories have been proposed, including:

1) glycolysis and subsequent fermentation is a faster way to produce ATP when resources are low, which gives tumor cells an evolutionary advantage over the surrounding tissues; 2) the flux through glycolysis and lactate formation provides more precursors for biomass production and therefore aids in rapid proliferation;[36] 3) the reprogrammed metabolism is not a hallmark of cancer cells, but the source of cancer development itself. No matter which theory is true, the difference in energy production needs to be addressed if one is to study energetic markers in a cancer cell model such as the herein used HepG2 cell line.

1.2.4. Nutrient and Energy Sensing

Cellular processes are tightly regulated at all levels, and every process has a certain energy requirement. Cells manage to maintain their status, through mechanisms that sense the energy requirement and relate it to nutrient capacity and energy availability. In case of an imbalance, metabolic pathways can then be stimulated as needed. How effective an organism can sense fluctuations in environmental nutrient levels determines evolutionary selectivity.[37] Two processes need to be considered for nutrient sensing: a nutrient binding to its receptor, which can propagate the information of binding, and the ability to detect and sense levels of a compound that directly reflect nutrient concentration.[37] Sensing mechanisms vary for the main three macronutrients, and vary for intra- and extracellular detection.

When amino acids are scarce, empty tRNAs (the molecules that shuttle amino acids to ribosomes for peptide synthesis) can be detected intracellularly by the general control nonderepressible 2 protein (GCN2), which can inhibit translation. Lipids, especially fatty acids, bind to G-protein coupled receptors and set off the release of hormones and other messenger molecules.[37] Glucose sensing has a very complex regulatory framework, due to its importance as the main energy source in mammalian systems. A multitude of mechanisms exist to assess glucose levels inside and outside of a cell. One of them is the activity of glucokinase (GCK), which is only active in a state of intracellular glucose abundance and therefore has the capacity to signal glucose levels. For extracellular levels, the glucose transporter GLUT2 can import glucose into the cell when the concentration is high, but exports out of the cell when levels are lower than the intracellular concentration. AMP-activated protein kinase (AMPK) and mTOR are universally seen as sensors of cellular energy and nutrient status in eukaryotic cells.[38,39] AMPK is sensitive to the ratio of

AMP:ATP and influences catabolic and anabolic processes to both, reduce or increase, ATP production or expenditure.[38,40] Phosphorylation of the kinase also modulates its activity to an increase of more than 100-fold compared to the unphosphorylated form. On top of that, influx of calcium ions into the cytosol increases AMPK activity and triggers production of ATP.[41] Extensive reviews on the activation and signaling involvement of AMPK have been published by Hardie.[41 – 45] Similarly to AMPK, mTOR has been suggested as a direct sensor of intracellular ATP as its activity decreases with the levels of the nucleotide.[39] Amino acids, fatty acids, metabolites from nutrient catabolism reactions, and some of their enzymes also alter its activity;[39] therefore, mTOR additionally qualifies as a nutrient sensor. Since both, AMPK and mTOR are highly involved in nutrient and energy sensing, it is no surprise that their pathways cross and affect each other's targets and activities. Their signaling reach is not confined to nutrients and energetics though, both molecules have critical impact on other functional domains, including cytoskeletal dynamics, cell growth, and programmed cell death.[46] Additionally, signaling around mTOR and AMPK are essential for the intracellular communication of nutrient levels regarding all three macronutrient classes.[37]

A step removed from nutrient awareness is the capability to use these nutrients to produce energy, or to decide to store them if enough energy is available at a given moment. However, that requires the systems to be able to sense energy demand and energy availability, especially in terms of ATP, if a system wants to remain within acceptable metabolic boundaries. A relatively large fraction, almost 30%, of cellular ATP is devoted to ion pumps, which maintain ion homeostasis under normal conditions. However, it has been shown that under anoxia, the fraction increases to 74% and expenditure on other processes decreases, suggesting a hierarchical allocation of energy resources to the most important activities. ATP itself can act as a regulator for certain pathways, for example: high concentrations of ATP inhibit phosphofructokinase, the first enzyme in glycolysis, which reduces the production of ATP. Rising levels of AMP on the other hand, a sign of an increase of ATP hydrolysis from energy-requiring reactions, activate the same enzyme, which increases the rate of glycolysis and leads to an increase in ATP production through oxidative phosphorylation. The same is true for AMPK, which decreases in activity with rising levels of ATP.[47]

1.3. Apoptosis and Autophagy

Eukaryotic organisms are made up of many cells, each with checks and balances to ensure energy supply, upkeep of function, and proliferation. However, most cells have a limited lifespan which is tightly regulated by programmed cell death (PCD) pathways. They ideally regulate a cell's downfall intracellularly to go by in an orderly fashion without dragging the immediate environment down the same path. Two primary forms of these PCDs have been identified based on distinctly different morphological features[48]: apoptosis (type I) and autophagy (type II). Anoikis is also categorized as a PCD, however, this form of cell death occurs only when a cell detaches from the surrounding tissue and undergoes a process very similar to apoptosis due to the loss of connectivity.[49]

1.3.1. Apoptosis

Apoptosis is the main form of programmed and genetically encoded cell death, that is especially important in early stages of mammalian development, where organs and extremities form, that would otherwise be shapeless.[50] Balanced cell proliferation and cell death upholds the status quo in a biological system. Irregular apoptosis has been connected to a plethora of diseases, the most prominent being cancer, where the apoptotic programs are turned off or inhibited. Morphological changes that are characteristic for apoptosis, and easily studied under a microscope, include the condensation of chromatin and a shrunken nucleus, formation of blebs and apoptotic bodies, and degraded plasma membranes.[49–51]

Initiation can either be extrinsic, intrinsic, or granzyme related.[48,51–53] If an extracellular molecule from the TNF family (tumor necrosis factor) binds to their so called "death" receptors on the plasma membrane, a death-inducing signaling complex (DISC) is formed, and inactive procaspases (caspase 8 or 10) can dimerize and activate each other by cleavage into caspases.[49,54] Intrinsic apoptosis can be triggered in nuclear, reticular, lysosomal, and plasma-membrane pathways,[22] which all seem to converge at the point of Bax/Bak pore formation at the mitochondrial membrane. Bax and Bak proteins belong to a subfamily of pro-apoptotic molecules in the family of Bcl-2, which play an integral part in apoptosis.[51] Depending on the number of Bcl-2 homology domains, members of different subfamilies can have quite the opposite effect on

mitochondrial membrane integrity. Bcl-2 for example inhibits Bax and Bak interaction by binding to them.[48,49,51,55] In the case of Bax and Bak interaction, insertion into the mitochondrial membrane leads to pore-like channel formation. With the formation of these pores on the outer mitochondrial membrane, the permeability increases drastically.[53] Thus, species such as cytochrome c are released into the cytosol.[52] Once in the cytosol, cytochrome c activates caspases (caspase 9), which ultimately lead to cell death.[51] Caspases are the best option, if one wants to study a compounds effect on apoptosis, although it is important to distinguish between the initiating caspases (2, 8, 9, 10) and the effecting caspases (3,6,7), since their activity depends on the timing in the program of apoptosis.[48]

1.3.2. Autophagy

The term autophagy very literally describes a biochemical processes, where, in accordance with the word's origin, a cell digests itself. When nutrients are less abundant, cellular constituents can be broken down to yield resources for energy production until nutrients are available again.[56,57] Several factors indicate that the recycling capacity in autophagy is an important part of homeostatic control and experiments showed a dependence on autophagy for cell survival.[58] However, autophagy can also lead to cell death.[53,55,56] During autophagy, cytoplasmic material is enclosed by an isolation membrane[59] into a membranous organelle, the autophagosome, which can fuse with lysosomes to initiate digestion of the respective material.[57] This way, autophagy provides resources for biosynthesis and cleans up the cytosol.[55,57] The autophagosomes are observable by microscopy as vacuole-like, double-membraned bodies.[49] The mitochondria plays a vital role as the origin of autophagosomal membrane in addition to its role in activating autophagy. Autophagy occurs constantly at low levels in the cell, however, activity is increased at certain conditions such as during nutrient deprivation. Eleven autophagy-related (Atg) genes haven been identified in mammals, prominent ones being Atg8 (LC3) and Atg6 (Beclin1), which initiate the formation of autophagosomal vesicles.[48] If the mitochondrial production and the cellular concentration of ATP is low, AMPK is activated and can either phosphorylate the serine/threonine-protein kinase UNC-51 like kinase 1 (ULK1) directly to upregulate autophagy, or inhibit the mechanistic target of rapamycin complex 1's (mTORC1) inhibition on ULKs by inhibitory phosphorylation of mTORC1s two regulators (tuberous sclerosis complex 2 [TSC2] and regulatory-associated protein

of mTOR [RAPTOR]), which also leads to a higher rate of autophagy.[15]As much as scientists want to classify and bring biochemical processes in order, it is not possible to completely separate autophagy and apoptosis. They are not two distinct routes of action, but rather intimately linked.[55] Efforts to generalize the exact roles and mechanisms have also not been successful, as it appears that the interplay between them is highly complex and varies in cell types, tissue types, and individuals.[55] Factors that affect autophagy are manifold, greatly relate to nutrient availability (mTOR, AMPK)[46,48,60] and the associated hormone signaling[57] and are also linked to apoptosis,[48] which has been reviewed by Fan and Zong (2013). To quote their closing remarks:

> *"autophagy clearly has both pro-survival and pro-death functions, and this is largely dependent on the cell's ability to die by apoptosis […] When cellular damage is too severe, apoptosis kicks in to eliminate irreparable cells to maintain tissue homeostasis. In cells with defective apoptosis, autophagy may serve as a backup strategy for cellular demise or as a mechanism to promote other forms of cell death, such as necrosis."* [55]

This interwoven relationship poses a challenge to anyone who wants to study the effects of a compound on autophagy, since markers should ideally be as exclusive to the investigated phenomenon as possible. In the field of autophagy research, scientists aim to differentiate between symptoms that possibly hint to autophagy and actually increased flux through the entire autophagy pathway.[61] An estimate of LC3 (Atg8) is often used to determine induction or inhibition of autophagy.[48] AMPK and mTOR phosphorylation can offer information about the activity and probably the effects on autophagy.[61]

1.4. Hormetic Compounds

Plant-based substances (phytochemicals) are commonly investigated for hormetic behavior. These compounds - often polyphenolic extracts[62] that include phytoestrogens, phytosterols, carotenoids, chlorophyll, isothiocyanates,[63] and flavonoids[64] - do not hold any nutritional value, since they do not fall into the category of proteins, carbohydrates, or fats. Phytochemicals often display antimutagenic and anticarcinogenic properties,[65] which sparked the recommendation to ingest at least five servings of vegetables and fruit (sources of phytochemicals) in the hope that these aid in

the prevention, retardation, treatment, or cure of certain diseases, including cancer.[1,6,66] Interestingly, due to their biphasic behavior, hormetic substances can, at lower doses, protect cells from DNA damage and the subsequent development of cancer, and at higher doses, be used as therapeutic supplements against cancer due to their cytotoxicity.[3] Bits and pieces of the exact modes of action for phytochemicals are known, but the whole picture remains to be clarified,[5] and the question persists if they share a common mode of action in the concept of hormesis, or if each phytochemical triggers its own profile of distinct beneficial processes inside the cell.[66] Some common affected targets have been summarized by Murugaiyah and Mattson (2015), which include AMPK and mTOR.[1]

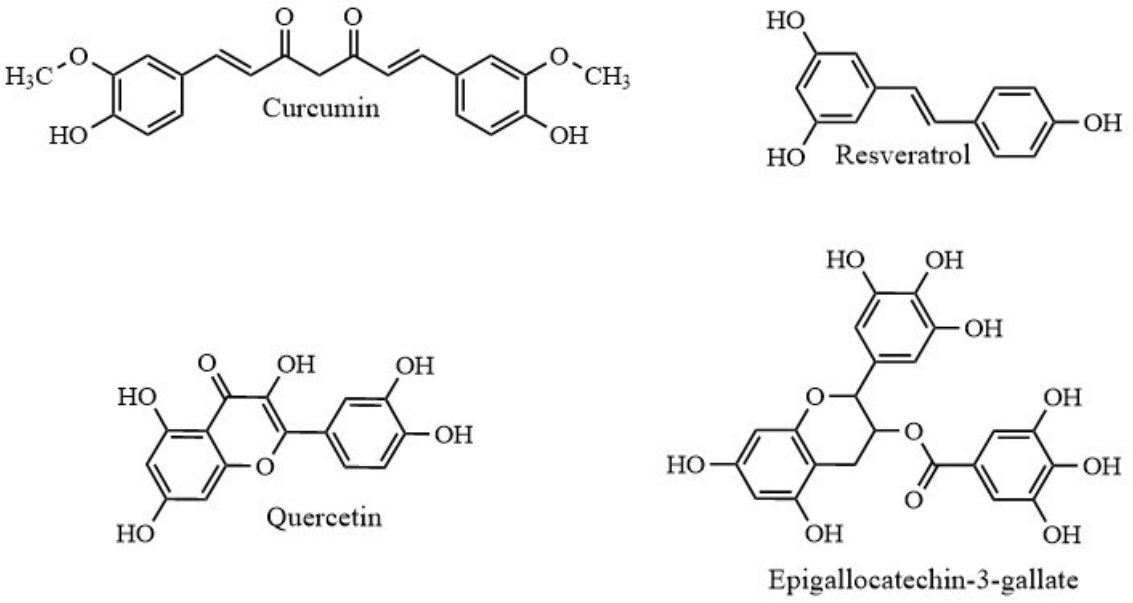


Figure 4| Chemical structures of curcumin, resveratrol, quercetin, and epigallocatechin-3-gallate.

1.4.1. Quercetin

Quercetin is a flavonoid derived from nuts, seeds, olive oil, vegetables, herbs, buckwheat, and teas, and is highly abundant in the human diet.[3,67–69] The main properties ascribed to quercetin are associated with ROS and their potential damage to tissue. As an electron donor to ROS[67] and a chelator for certain metal ions, quercetin achieves anti-oxidant results, and therefore has been shown to decrease the incidence of DNA damage and the chances of cancer development.[3] Additionally, quercetin can induce expression of the DNA repair protein 8-oxyguanine DNA

glycosylate (hOGG1)[67,70] and activates the antioxidant glutathione.[67,69] Induction of metallothioneins (MT) by quercetin has been reported to protect against heavy metal toxicity in a liver carcinoma model.[71] These properties are observed at doses below 40 µM,[3] although Kim *et al.* (2009) observe antioxidant effects up to 100 µM 30 minutes post-exposure. Tan *et al.* (2009) found that simultaneously decreased levels of the anti-apoptotic proteins survivin and Bcl-2 as well as increased levels of p53 and increased activity of caspase-3/-9 cause cytotoxicity in HepG2 cells, but their data did not support hormetic behavior.[72] There is no clear agreement on quercetin's hormetic properties, which is why the compound should be investigated more thoroughly.

1.4.2. Epigallocatechin Gallate

The green tea, black tea, and red wine component epigallocatechin gallate showed a beneficial antioxidative character in several studies,[73] and has additionally been found to have anti-carcinogenic, anti-angiogenic, as well as anti-proteolytic features,[74] especially in cases of ethanol and hypoxia induced damage, where epigallocatechin gallate ameliorated the effects.[73,74] In HepG2 cells, induction of autophagy has been assigned to an exposure of 40 µM of catechin,[75] while Shen *et al.* (2014) reported a decrease in autophagy induction of approximately 25% after a 24- and 48-hour exposure of 87 µM and by 45% after 78 hours.[76] Additionally, 174.5 µM and 262 µM inhibited metabolic activity in the same cell line by 40 – 45% after 24 hours, 65% after 48 hours, and 70% after 72 hours, respectively. Hormesis tends to be observed at lower concentrations than the ones listed here, so an expansion of the dosing range into smaller concentrations might reveal hormetic behavior. For a review of epigallocatechin gallate's origin, bioavailability, see Roberto *et al.* (2016).[77]

1.4.3. Curcumin

Derived from turmeric, curcumin is a yellow to orange colored powder that has been extensively studied in the last years, especially with respect to the prevention and treatment of cancer.[78] Cellular effects found *in vitro* and *in vivo* in varying cell lines include the induction of mitochondria mediated apoptosis,[78–80] autophagy,[80,81] disruption of mitochondrial membrane potential,[79] increase in calcium ion concentration,[79] antioxidant characteristics through binding of

Nrf2[82] and prooxidant characteristics at higher doses. In HepG2 cells, Wang *et al.* (2011) observed a decline in cell viability response by 20% starting at 5 µM, almost linearly decreasing until one-fifth of the control values at 20 µM.[79] In a different paper, Qian *et al.* (2011) found a 20% increase in cell viability after a 12-hour exposure to 5 µM, while all other exposures of 24 and 36 hours to 20 and 50 µM either did not affect the cell viability compared to controls, or decreased cell viability. Therefore, curcumin might show hormetic behavior.

1.4.4. Resveratrol

Resveratrol is a polyphenol found in peanuts and grapes, predominantly, that has been thoroughly studied for antiproliferative, chemo-preventive, cardio-protective, immune system improving, antiaging, gut health regulating, and antioxidant effects.[83–87] Marcsek *et al.* (2007) found slight, but not significant, increase in cell proliferation after exposing HepG2 cells to 0.1 to 100 µM for 24 hours. After 48 hours, cell viability was unaffected by concentrations up to 12.5 µM but declined from there to about 50% (100 µM), and 72-hour exposures gave similar results.[83] Plauth *et al.* (2016) on the other hand found increased cell viability up to 100 µM in the same cell line, although exposure time was only 16 hours.[84] An increase in signal from MTT assays by 20% for resveratrol concentrations between 30 and 50 µM after 24 hours was reported, but the effect did not last for an exposure of 48 hours.[88] Such proliferative effects have been observed in a variety of human cancer cell lines and preventive effects for other diseases, such as Alzheimer's or gastric ulcers, have also been described.[89] Additionally, co-exposure of resveratrol with, for example, matrine or formaldehyde, decreased the detrimental effects of the cytotoxic substances.[83,90] Between the three previously cited papers, it is clear that resveratrol causes a hormetic adaption, however, exact concentrations vary mainly because the exposure times are not matched. A more detailed study into these two aspects should be able to resolve uncertainty.

2. Experimental Design, Methods, and Materials

2.1. Experimental Design

This work aims to clarify the concentration and exposure duration dependence in hormetic behavior for several compounds. For this thesis, four compounds were chosen, based on literature review, and human hepatocyte carcinoma cells were exposed to varying concentrations. The first set of experiments was meant to screen these four compounds for the best concentration range to observe potentially biphasic behavior. Depending on the results, compounds were then selected for additional studies involving a series of different exposure times to address the duration dependence. Concentration ranges were kept constant throughout this part to ensure complete comparability of data.

2.1.1. In vitro Model

For the herein described experiments, a human hepatocyte carcinoma cell line (HepG2) was chosen as the *in vitro* model. The mammalian liver hosts major phase I and phase II metabolism of xenobiotics, and offers detoxification capabilities, but can also yield metabolites with increased harm for the system. Therefore, metabolic experiments from this cell line can deliver important information about the pharmacokinetic nature of phytochemicals. In addition, the cell line provides insight into liver carcinoma-specific metabolism and how the investigated compounds can affect cancer cell survival.[5] Although single-cell experiments give a more exact picture on metabolic changes,[12] measurements from a cell population can at least suggest trends and point into the right direction for further studies.

2.1.2. Cell-based Assays

Four substances were chosen due to their previously reported hormetic behavior or their potential for hormetic behavior: Curcumin, epigallocatechin gallate, resveratrol, and quercetin. The initial experiments aim to characterize appropriate concentration ranges, optimize assay conditions, and rule out compounds that give inconsistent results. Subsequent experiments monitor the metabolic activity and plasma membrane degradation across the determined concentration range. Exposure

duration dependence was examined through measurements six, twelve, 24, 48, and 72 hours after a one-time dose of the compound.

Concentration ranges investigated were: 0.01 - 500 µM curcumin and epigallocatechin gallate, 0.01 - 2000 µM resveratrol and quercetin. Quercetin's effects greatly depend on the route of metabolism in the body, which is vastly branched.[3] Isorhammetin and tamarixetin are two well-studied metabolites, but for the purpose of this study, pure quercetin was used, since it appears to have the largest anti-oxidant effect.[3] Based on Lüer *et al.*'s (2014) demonstration that synthetic and natural curcumin have the same cellular effects, synthetic curcumin was employed in experiments.[91] While resveratrol exists in three forms in red wine (trans-, cis-, and dihydro-resveratrol), trans-resveratrol makes up the predominant constituent, which is why that species was chosen for investigation.[92] Epigallocatechin 3-gallate was employed for similar reasons (abundance in tea) and will further be referred to as epigallocatechin gallate.

Effects on cell viability after an exposure of 24 hours are assessed with the widely used MTT assay. Active cellular metabolism in live cells is commonly measured making use of MTT tetrazolium (3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide), which is converted to formazan by reducing molecules inside the cell.[93] The exact mechanism is not known yet, but it is assumed that MTT enters the cell through endocytosis and is reduced by several substrates, including succinate, malate, glutamate, NADH, and NADPH.[93] Liu *et al.* (1997) showed, however, that the reduction of MTT does not only rely on active mitochondria, as has been widely stated. Therefore, this assay merely measures metabolic production of reducing substrates. The amount of formazan increases linearly with cell number, presuming their metabolism is the same. Varying metabolic rates, for example in cells at different stages, can influence linearity and skew results.[94] MTT is usually dissolved in buffer, such as PBS, at a concentration of approximately 5 mg/mL[95] and diluted ten-fold in the assay well. Formazan accumulates as a purple crystalline precipitate over several hours; therefore, plates need to be incubated at 37 °C to increase sensitivity and signal stability. The blue-purple crystals are then solubilized, with solutions ranging from isopropanol and dimethylsulfoxide to preparations of SDS in DMSO,[94] and the relative concentration of formazan can be measured through absorbance at 570 nm. Assay kits supplying an MTT solution and detergent are commercially available.

Exposure effects leading to cell death through plasma membrane integrity are studied with ethidium-D1 homodimer When cells undergo cell death after a xenobiotic insult, the cell membrane is found to be compromised, which equals an increase in premeability.[96] This can be used to label dead cells with a - in principle - cell impermeable compound, such as ethidium-D1 homodimer, which binds to nucleic acid once it entered into the cytosol of a damaged cell.[97] The free nucleic acids is another sign of a cell moving towards cell death, especially during coordinated cell death (apoptosis), since the nucleus undergoes sequestration and the DNA is broken down.[49] The homodimer chelates the double stranded nucleic acids[98] and exhibits a red fluorescence[99] that can be measured at excitation/ emission wavelength 530/645 nm.

2.2. Methods and Materials

2.2.1. Materials

HepG2 cells were acquired from ATCC (ATCC® – HB-8065™). Cell culture media was prepared from Dulbecco's Modified Eagle's Medium (Sigma-Aldrich) in deionized water from a EMD Milipore DirectQ Water System. D-(+)-glucose, sodium pyruvate, L-glutamate monosodium hydrate, and sodium bicarbonate were purchased from Sigma-Aldrich. 100X solutions of streptomycin and penicillin were acquired from Corning, HyClone fetal bovine serum defined from Thermo Scientific and HEPES from Fisher BioReagents. 0.05% trypsin with 0.53 mM EDTA (1X), Costar polystyrene tissue culture treated clear flat bottom 96 well plates (low evaporation lid), Costar polystyrene tissue culture treated black with clear flat bottom 96 well plates, and T75 cell culture flasks with vented caps were procured through Corning. HPLC grade methanol was purchased from EMP, DMSO from Fisher BioReagents, 0.4% trypan Blue from MP, ethidium homodimer-1 from Invitrogen Molecular Probes, and the TACS® MTT Cell Proliferation Assay Kit from Trevigen. Quercetin, (-)-epigallocatechin gallate, and trans-resveratrol, were obtained from Cayman Chemical Company, while curcumin was purchased from Acros Organics.

2.2.2. Cell Culture

HepG2 (human hepatocellular carcinoma-derived) cells were cultured in cell line specific medium (DMEM, 2 g/L D-glucose, 10% fetal bovine serum, 100 mg/mL streptomycin, 100 U/mL penicillin, 1 mM sodium pyruvate, 2 mM L-Glutamate, 5 mM HEPES, and 24 mM sodium bicarbonate) and maintained at 37 °C in humidified, 5% CO_2 atmosphere. Cells were passaged at 70% - 90% confluence, using trypsin EDTA as lifting agent. Cell density was determined after trypan blue staining on a Reichert Bright-Line® Improved Neubauer hemocytometer in 10 µL of a 1:1 dilution of cell solution in staining reagent (20 µL each). For all assays, approximately 40 000 cells/100 µL were seeded into 96 well plates (clear for absorbance assays, black with clear bottoms for fluorescence) in indicator-free medium and allowed to reattach for 24 hours before dosing.

2.2.3. Dosing

Stock solutions of all compounds were prepared in DMSO in accordance with the substances' solubility: 10 mM and 50 mM curcumin, 50 mM epigallocatechin gallate, 100 mM resveratrol, and 100 mM quercetin.

100X dosing solutions were freshly mixed by diluting appropriate amounts of stock solution in DMSO, which were subsequently diluted 100-fold in cell culture medium to yield the final dosing concentrations. Curcumin: 0.01, 0.1, 0.5, 1, 5, 10, and 50 µM. 100 and 500 µM dosing solutions were created by directly diluting the 10 mM or 50 mM stock solution 1:100 in cell culture medium. Epigallocatechin gallate: 0.01, 0.1, 1, 5, 10, 25, 50, 70, 100, 150, 200, 250, 300, and 400 µM. 500 µM dosing solutions were created by directly diluting the 50 mM stock solution 1:100 in cell culture medium. Quercetin and resveratrol: 0.01, 0.1, 1, 5, 10, 25, 50, 70, 100, 200, 400, 500, 600, and 800 µM. 1000 µM dosing solutions were created by directly diluting the 100 mM stock solution 1:100 in cell culture medium, 2000 µM dosing solutions were created by directly diluting the 100 mM stock solution 1:50 in cell culture medium. All dosing solutions had a final DMSO concentration of 1% or 2%. Dosing solutions used in different assays may not include all listed concentrations and can vary between plates.

24 hours after seeding, spent cell culture medium was aspirated and discarded, and cells were supplied with 100 µL fresh culture medium in the form of the dosing solutions. 96 well plates were

either incubated for the respective exposure time at 37 °C in humidified, 5% CO_2 atmosphere or, for real-time measurements, covered with plate tape and kept at 37 °C in the plate reader.

2.2.4. Assays

All plates were evaluated using a Tecan InfiniteM100 plate reader (Tecan US, Raleigh, NC) and Tecan i-control 1.10 software.

2.2.4.1. MTT Assay

Cell viability assays were performed using the TACS® MTT Cell Proliferation Assay Kit (Trevigen) per manufacturer's protocol. Briefly, at the end of the respective exposure time, 10 µL/well MTT reagent (3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) were added. After six hours of incubation at 37 °C, 100 µL detergent were dispensed in each well, the plates covered in aluminum foil and stored in a dark cupboard for twelve hours. Absorbance was then read from the bottom at 570 nm. Controls for this assay included untreated 1% and 2% DMSO vehicle controls (where necessary), as well as blank controls without cells at each concentration for background absorbance, since some substances showed an increased absorbance with increased concentration. Control and experimental wells were treated identically throughout the assay. Average absorbance values were corrected for background absorbance from the blank controls and normalized to untreated vehicle controls wells.

2.2.4.2. Plasma Membrane Degradation Assay

Plasma membrane degradation assays were performed using ethidium-D1 homodimer. 1 mg was dissolved in 20% aqueous DMSO to a concentration of 2 mM. After the respective incubation period, 100 µL of 4 µM ethidium D-1 homodimer in culture medium were added to each well for endpoint experiments and kept at 37 °C in humidified, 5% CO_2 atmosphere for 20 minutes. Fluorescence was measured from the bottom with excitation at 530 nm and emission at 645 nm. Controls for this assay included untreated 1% DMSO vehicle controls, blank controls without cells at each concentration, and control wells of 100% dead cells (removal of reaction mixture and

addition of 100 µL 70% methanol in medium 30 minutes prior to the end of the respective incubation period) for maximum signal. Control and experimental wells were treated identically throughout the assays. Average fluorescence values were corrected for background fluorescence through blank control values and normalized to maximum signal responses (unless otherwise specified).

2.2.5. Data Evaluation and Statistical Analysis

Data from cell-based assays were exported and analyzed with Prism 5.03 (GraphPad), values are reported as the mean ± standard error of the mean. For cell viability assays, values were first corrected by subtracting the corresponding blanks and then normalized to the vehicle control (no treatment, assumed 100% live). For plasma membrane degradation assays, values were first corrected by subtracting the corresponding blanks and then normalized to the dead control (70% methanol 30 minutes prior to the end of the exposure time, assumed 100% dead). Statistical significance was determined using one-way ANOVA with Bartlett's test for equal variances and Bonferroni's Multiple Comparison Test. The following notation is used to report p-values (unless otherwise specified): * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ compared to vehicle control; $^{\#}$ $p \leq 0.05$, $^{\#\#}$ $p \leq 0.01$, $^{\#\#\#}$ $p \leq 0.001$ compared to the previous concentration, $^{\circ}$ $p \leq 0.05$, $^{\circ\circ}$ $p \leq 0.01$, $^{\circ\circ\circ}$ $p \leq 0.001$ compare to the previous exposure time.

3. Results and Conclusion

3.1. Substance Screening

Human hepatocyte carcinoma cells (HepG2) were exposed to varying concentrations of epigallocatechin gallate, resveratrol, quercetin and curcumin for 24 hours. Effects on metabolic activity and plasma membrane integrity were assessed using MTT (3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) and ethidium-D1 homodimer, respectively. Results are summarized in Figure 5 and Table 1. Substances were screened for hormetic behavior, e.g. an increase in metabolic activity above controls level and a simultaneous decrease in plasma membrane degradation at lower concentrations; a decrease of metabolic activity associated with an increase in plasma membrane degradation at higher concentrations.

3.1.1. Epigallocatechin Gallate, Quercetin, and Curcumin

Effects of epigallocatechin gallate treatment on metabolic activity at concentrations up to 10 µM were unchanged from basal control levels. From 25 to 500 µM, the metabolic activity increased in the mean by 28 to 86%. However, variability in the four independent experiments was high and the variance in means did not show a significant change at higher concentrations. Additionally, no decrease in metabolic activity was observed. Plasma membrane degradation results showed a decrease compared to vehicle controls at 25 and 50 µM. Data points from higher concentrations consistently gave invalid results and are not included here. In theory, a decrease in plasma membrane degradation in association with an increase in metabolic activity matches hormetic behavior. Nevertheless, variability was considerably too high, especially in MTT results, to recommend epigallocatechin gallate for further duration dependence experiments. Zapf *et al.* (2015) concluded that an exposure of 4.4 µM over twelve, 24, and 48 hours did not decrease cell viability in the same cell line, which the herein presented results support.[100] However, Shen *et al.* (2014) reported decreased metabolic activity from 87, 174.5, and 262 µM (25%, 45%, 45%, respectively) after 24 hours of exposure, [76] which is partially matched by other studies,[101] but was not reproduced in the presented experiments. In fact, our results rather suggest an increase in metabolic activity for concentrations above 100 µM. The typical hormetic dose response behavior was not observed for the investigated cell line. Naponelli *et al.* (2015) reported that

epigallocatechin gallate induces endoplasmatic reticulum stress in prostate cancer cells, but the cells were able to overcome this stress at least between the first six to twelve hours. After that, 76 µM induced anoikis, and 316 µM induced cell death.[58] In its nature as a catechin, epigallocatechin gallate is presumed to owe its beneficial character to the activation of Nrf2, which increases activity of phase II enzymes.[6] Calabrese *et al.* (2010) state that various pathways can be influenced by this molecule, including protein kinase C, Bcl-2 family members, and antioxidant

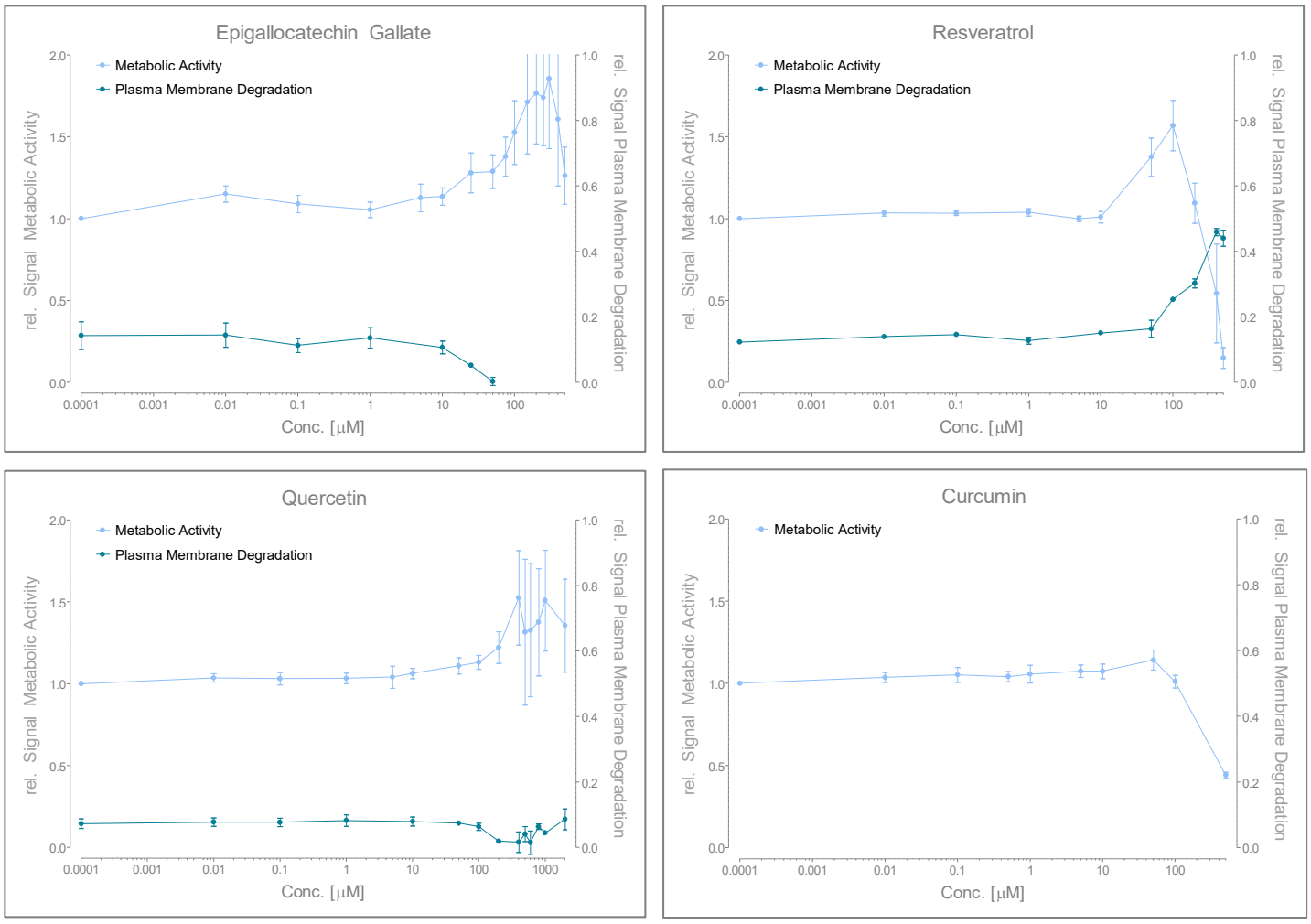


Figure 5| Metabolic activity and plasma membrane degradation after 24-hour exposure to epigallocatechin gallate, resveratrol, quercetin, and curcumin.

HepG2 cells were exposed to varying concentrations of epigallocatechin gallate (top left), resveratrol (top right), quercetin (bottom left), or curcumin (bottom right) for 24 hours. Effects on metabolic activity were assessed using MTT, effects on plasma membrane integrity were assessed using ethidium homodimer-1. Data shown represent the mean and the standard error of the mean from four independent experiments. Experimental values were measured in duplicate, triplicate, quadruplicate, or sextuplicate.

enzymes.[102] Ye *et al.* (2012) found activated SIRT1 signaling (an energy sensor for NAD^+) when PC12 cells - a rat adrenal gland cell line - were exposed to a cytotoxic compound (MPP^+) alongside 10 µM epigallocatechin gallate, which increased cell viability markedly compared to treatment with the cytotoxic compound alone.[103] The green tea extract undoubtedly exerts positive effects, but is most likely not a hormetin.

Similar to epigallocatechin gallate, exposure to quercetin did not trigger a change in cellular behavior at low doses up to 10 µM. From 50 µM to 2000 µM, trends in metabolic activity and plasma membrane degradation complemented each other, however, none of the increases or decreases are statistically significant and the standard error of the mean at higher concentrations was considerably high with regards to metabolic activity. Additionally, quercetin precipitated out of the culture media for concentrations above 500 µM soon after dosing, therefore the true concentration in media is unknown. Considering that, no evidence has been found that quercetin exerts hormetic behavior below 800 µM and quercetin was not included in additional experiments. While there were observable trends associated with metabolic activity and plasma membrane integrity, the results mostly do not agree with literature values. Tanigawa *et al.* (2008) found a decrease in MTT response after 24 hours from 20 µM,[104] while Tan *et al.* (2009) observed a considerable decrease in MTT response after 24 hours starting at 12.5 µM and an even bigger effect after 48 and 72 hours.[72] However, Weng *et al.* (2011), mention no decrease in MTT response up to 50 µM,[71] which these experiments support. The problem of quercetin stability in culture media has been addressed previously,[68] finding that solutions of quercetin above 80 µM start to precipitate out at a pH of 7.4. Therefore, literature values should be taken with a grain of salt if they claim results for anything higher than 80 µM, as is the case for the results presented here and in Pietsch *et al.*'s 2011 publication.[66]

No statistically significant change in metabolic activity was observed from curcumin exposure, although the data displays a slight upward trend at 50 µM before returning to control level at 100 µM and subsequently decreasing to less than 50% at 500 µM. Measurements involving plasma membrane degradation were challenging, since curcumin posed an experimental challenge due to its absorption at the experimental wavelength which has been previously discussed.[105,106] Several control groups (blanks at each concentration including dosing vehicle, blanks of cells with dosing reagent at each concentration without assay reagent, and dead controls for maximum signal) and

data manipulations were employed assuming to account for the analyte signal, but data evaluation did not yield usable values. Therefore, curcumin was no longer pursued. Literature so far has reported hormesis-like effects on HepG2 cells at low doses of 5 µM after twelve hours of exposure, while the same paper does not see hormetic behavior after an exposure of 24 hours, which our results support.[81] Besides its multitude of positive effects on the human organism, it appears that curcumin does not act like a hormetin, at least not in the investigated liver cancer model. Adenocarcinoma cells (MCF-6) on the other hand seem to increase proliferation up to about 75 µM after 24 hours, with no significant decrease in proliferation up to 100 µM.[68] The flavonoid structure allows for a potential inhibition of tyrosine nitration, which would relief excessive nitrosative stress.[64] Concentrations higher than 40 µM exert cytotoxic effects based on quercetin acting as a ROS itself.[3] Data from breast cancer cells (MDA-MB-231) has been presented,[107] however, the authors did not see a significant decrease in cell viability until cells were exposed to 100 µM for 24 hours.[107] Vargas and Burd's findings (2010) suggest a mitochondrial and a death-domain apoptotic pathway.[3] Pietsch *et al.* (2011) found that doses of 100 and 200 µM induced proliferation in *C. elegans*, but reduced the same metric at a dose of 250 µM.[66]

3.1.2. Resveratrol

Exposure to resveratrol exhibited a clear trend in metabolic activity and plasma membrane degradation. Doses below 10 µM did not change the cellular response compared to basal level in both assays. An increase in metabolic activity of 38% and 57% stood out for 50 µM and 100 µM, respectively, although not statistically significant, while the level of plasma membrane degradation steadily increased from a control level of about 15% (compared to 100% dead) to 25% for 100 µM and even almost 45% for the highest doses of 250, 400, and 500 µM. The change in plasma membrane integrity was statistically significant for 100, 250, 400, and 500 µM. 500 µM also significantly decreased metabolic activity to less than 15% of basal level. In the presented preliminary experiments, resveratrol did not show statistically significant hormetic behavior, however, the standard error of the mean was smaller and data more consistent than other compounds in question. Therefore, HepG2 cells were exposed to varying concentrations of resveratrol for six, twelve, 24, 48, and 72 hours.

Table 1| Summary of statistically significant changes in metabolic activity and plasma membrane integrity by compound concentration compared to untreated vehicle controls and the previous concentration.

	Compared to Untreated Vehicle Control		Compared to Previous Concentration	
	Concentration [μM]	* p≤0.05 ** p≤0.01 *** p≤0.001	Concentration [μM]	# p≤0.05 ## p≤0.01 ### p≤0.001
Epigallocatechin Gallate				
Metabolic Activity	No statistically significant changes		No statistically significant changes	
Plasma Membrane Integrity	50 & 75	**	No statistically significant changes	
	100 - 500	***		
Resveratrol				
Metabolic Activity	500	***	No statistically significant changes	
Plasma Membrane Integrity	100	**	100	#
	200 - 500	***	400	###
Quercetin				
Metabolic Activity	No statistically significant changes		No statistically significant changes	
Plasma Membrane Integrity	No statistically significant changes		No statistically significant changes	
Curcumin				
Metabolic Activity	500	***	500	###

3.2. Resveratrol: Concentration and Duration Dependence

Preliminary 24-hour exposure experiments in HepG2 cells showed promising results for hormetic behavior associated with resveratrol. To test concentration and exposure duration dependence, the same cell line was exposed to varying concentrations (0.1 – 500 μM) for six, twelve, 24, 48, and 72 hours and the aspects of metabolic activity and plasma membrane integrity were investigated. Results are summarized in Figures 6, 7, and 8, and Tables 2 and 3.

Figure 6 presents data from metabolic activity and plasma membrane degradation measurements for each time point. The panels clearly show an increase in metabolic activity for certain concentrations, while plasma membrane degradation mainly stayed at basal levels until concentration and exposure times increased.

In detail, cellular responses associated with metabolic activity were approximately the same after six and twelve hours, with an increase in metabolic activity caused by 50 and 100 µM by 30% and 85 – 89% of the basal control level respectively. Values obtained from 250 µM show a 10% higher

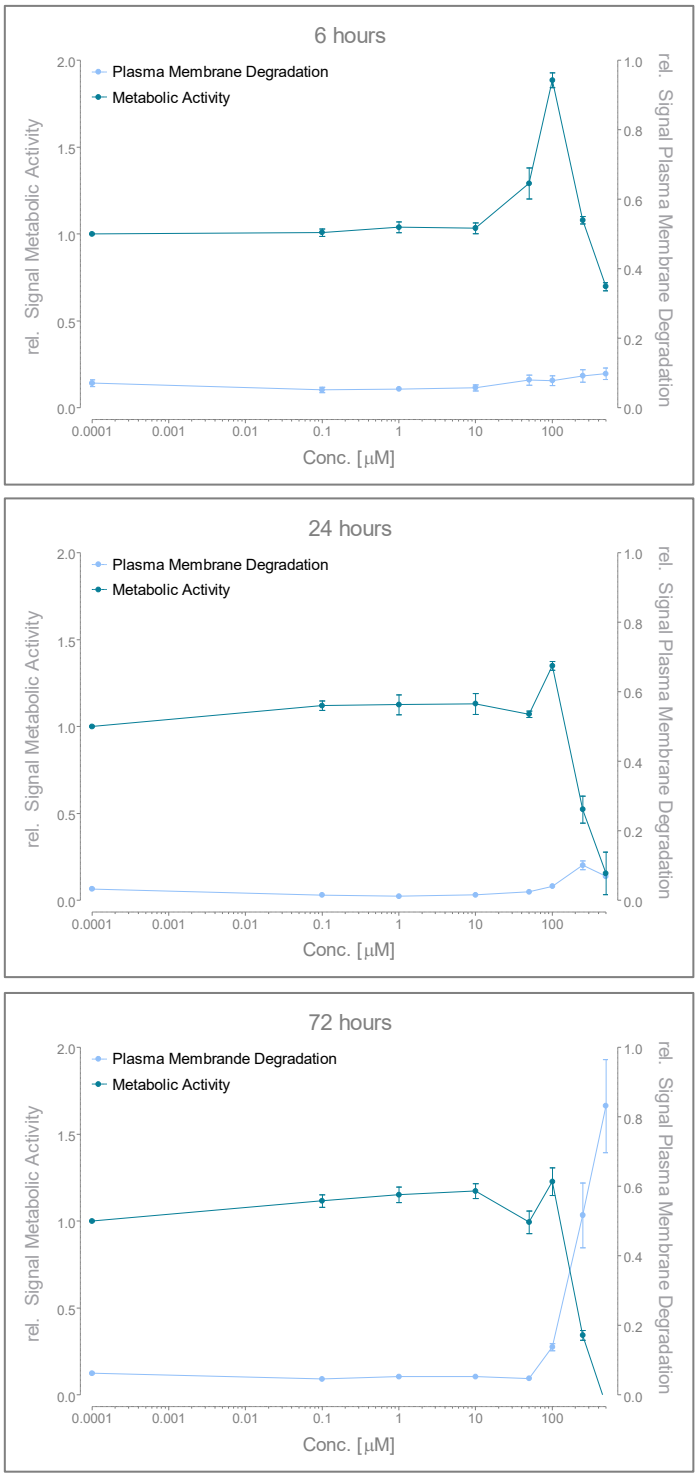


Figure 6| Metabolic activity and plasma membrane degradation after exposure of HepG2 cells to resveratrol for six, twelve, 24, 48, and 72 hours.

HepG2 cells were exposed to 0.1, 1, 10, 50, 100, 250, and 500 µM for six (top left), twelve (top right), 24 (middle left), 48 (middle right), and 72 hours (bottom). Effects on metabolic activity were assessed using MTT, effects on plasma membrane degradation were assessed using ethidium homodimer-1. Data shown represent the mean and the standard error of the mean from four to six independent experiments. Experimental values were measured in quadruplicate for blanks, triplicate for controls, and sextuplicate for experimental wells.

metabolic activity, but the variances in the mean were not statistically significant. 500 µM resveratrol considerably reduced cellular activity to approximately 75% of the untreated controls. Metabolic activity noticeably (and statistically significantly) changed between twelve and 24 hours. After 24, 48, and 72 hours, 250 µM doses inhibited metabolic activity to less than 50% on average, and 500 µM decreased to less than 15%. 50 µM did not increase the cellular response, but 100 µM did for all three later exposure times. The spike of 35% after 24 hours, 26% after 48 hours, and 22% after 72 hours was statistically significant. The disconnect between the early time points of six and twelve hours to the later time points of 24 to 72 hours is especially well presented in Figure 7 (left panel). The right panel in the same figure shows that from 50 to 500 µM, the cellular behavior follows the same trend, where the dose increases metabolic activity from 6 to 12 hours, and then decreases relative to the previous level from 24 to 72 hours. This change is statistically significant between exposure points from 12 to 24 hours at compound concentrations of 50, 100, 250, and 500 µM. It clearly shows that 100 µM has the largest initial influence on the cell, with the highest increase in activity. 50 µM has a comparable effect, but in a smaller magnitude. Interestingly, another trend can be observed at lower concentrations between 0.1 and 10 µM: with an increase in exposure time, the metabolic activity increased, which seems to be distinctly different than after six and twelve hours, but is not statistically different.

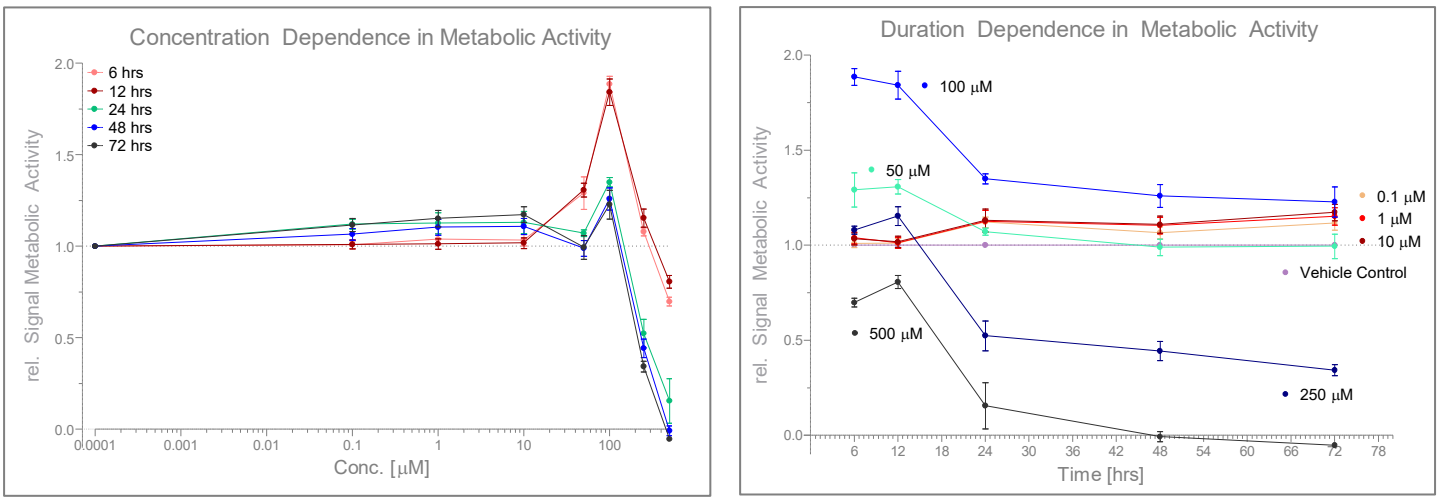
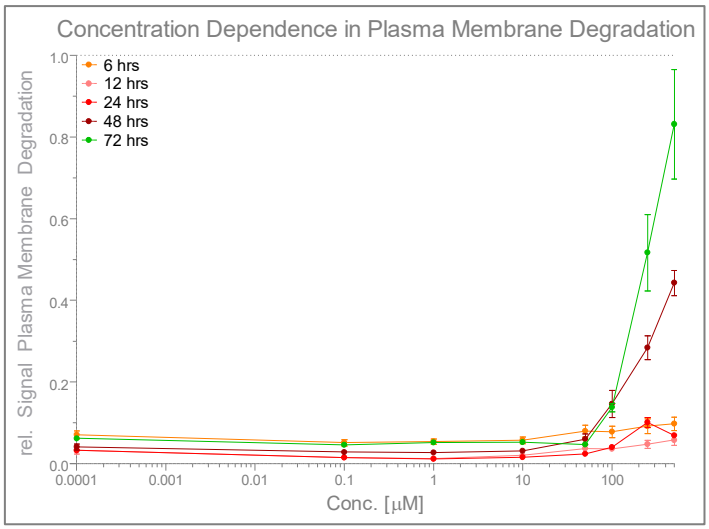


Figure 7| Metabolic activity of HepG2 cells exposed to resveratrol for 6 to 72 hours.

Comparison of the different concentrations (left) and exposure times (right). A clear change in metabolic activity was observed between twelve and 24 hours, especially in response to 50, 100, 250, and 500 µM. A slight increase over time is noticeable for lower concentrations from 0.1 to 10 µM. Data shown represent the mean and the standard error of the mean from four to six independent experiments. Experimental values were measured in quadruplicate for blanks, triplicate for controls, and sextuplicate for experimental wells.



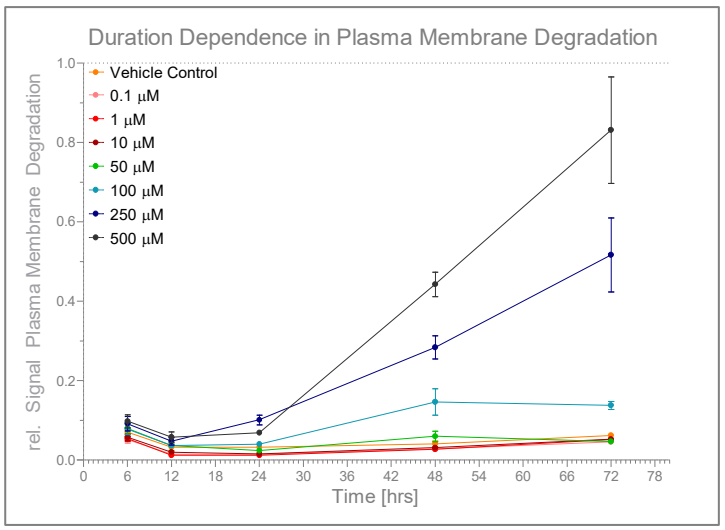


Figure 8| Plasma membrane degradation of HepG2 cells exposed to resveratrol for 6 to 72 hours.

Comparison of the different exposure times (right) and concentrations (left). Plasma membrane integrity was noticeably compromised after 48 hours of exposure to 250 and 500 µM. An initial decrease in plasma membrane degradation was observed for all concentrations. Data shown represent the mean and the standard error of the mean from four to six independent experiments. Experimental values were measured in quadruplicate for blanks, triplicate for controls, and sextuplicate for experimental wells.

The plasma membrane degradation data points a similar picture. No significant changes in plasma membrane integrity were observed for exposures duration up to twelve hours. After 24 hours, concentrations of 250 and 500 µM slightly increased plasma membrane degradation compared to the vehicle control, after 48 hours, 100, 250, and 500 µM displayed an increase to 14, 28, and 44% (of 100% dead). After 72 hours, 250 and 500 µM raised the degree of plasma membrane degradation to 50 and 83% in the mean (of 100% dead), respectively. Figure 8 (left) shows that from time point to time point, cellular behavior changed significantly from 24 to 48 hours concerning doses of 100, 250, and 500 µM, but also between 48 and 72 hours for 250 and 500 µM concentrations. When the data was plotted against time, all concentrations initially decreased plasma membrane degradation from six to twelve hours, although not statistically significant, it is a clear trend. Worth noticing is, again, the difference in behavior from low doses, which did not change plasma membrane integrity over time, and high doses, which evidently affected membrane degradation starting at 24 hours.

Table 2| Summary of statistically significant changes in metabolic activity and plasma membrane integrity between exposure times by resveratrol concentration.

		Compared to Previous Exposure Time	
	Exposure Time	Concentration [μM]	° p≤0.05 °° p≤0.01 °°° p≤0.001
Metabolic Activity	12 vs. 24 hours	50	°°
		100 - 500	°°°
Plasma Membrane Integrity	24 vs. 48	100	°
		250 & 500	°°°
	48 vs. 72	250 & 500	°°°

Table 3| Summary of statistically significant changes in metabolic activity and plasma membrane integrity after varying exposure times by resveratrol concentration compared to untreated vehicle controls or the previous concentration.

	Compared to Untreated Vehicle Control		Compared to Previous Concentration	
	Concentration [μM]	* p≤0.05 ** p≤0.01 *** p≤0.001	Concentration [μM]	# p≤0.05 ## p≤0.01 ### p≤0.001
6-hour Exposure				
Metabolic Activity	50 & 100	***	50	##
			100 - 500	###
Plasma Membrane Integrity	No statistically significant changes		No statistically significant changes	
12-hour Exposure				
Metabolic Activity	50 & 100	***	50 – 500	###
Plasma Membrane Integrity	No statistically significant changes		No statistically significant changes	
24-hour Exposure				
Metabolic Activity	100	*	250	###
	250 & 500	***	500	##
Plasma Membrane Integrity	250	***	250	###
	500	**	500	#
48-hour Exposure				
Metabolic Activity	100	**	100	##
	250 & 500	***	250 & 500	###
Plasma Membrane Integrity	100	*	250	##
	250 & 500	***	500	###
72-hour Exposure				
Metabolic Activity	100	*	100	#
	250 & 500	***	250 & 500	###
Plasma Membrane Integrity	250 & 500	***	250	##
			500	#

With the assumption that the metabolic rate across the sampled cell population is - on average - the same, an increase in metabolic rate from the MTT assay can be seen as an increase in cell proliferation (more metabolic activity equals more metabolically active cells). Four striking observations were made: 1) Low concentrations (0.1 to 10 µM) display no statistically significant change in metabolic activity or plasma membrane integrity, but with increasing exposure time, a trend of a subtle increase in cell proliferation (Figure 6, right panel) and a subtle decrease in plasma membrane degradation (Figure 7, right panel) can be associated with these doses. 2) Higher concentrations (50 to 500 µM) evoke the same trend in HepG2 cells, opposite to the one observed for lower concentrations: Cell proliferation decreases as the exposure time increases; plasma membrane degradation increases as the exposure time increases. 3) Over the time course of 72 hours, the cellular behavior changes for the doses of 50, 100, and 250 µM: During the first twelve hours, the three concentrations increase metabolic activity; after 24 hours, only 100 µM still raises the assay response above control level. The two duration groups (six and twelve hours, 24 to 72 hours) share almost the same response to resveratrol. Therefore, at a time point between twelve and 24 hours, something changes in the cellular metabolism. Plauth *et al.* (2016) investigated resveratrol's effect on antioxidants and metabolite couples' concentrations, and found a significantly changed intracellular redox environment 16 hours after dosing. 4) When comparing metabolic activity and plasma membrane degradation at 100 µM, one can see that both increase, especially with an increase in exposure time (Figure 6). This suggests that cellular metabolism increases but the cellular membrane is also increasingly compromised, which might either be rooted in heightened flux in pathways that combat cell death via plasma membrane degradation or the opposite, heightened flux in pathways that eventually cause the permeabilization.

The basis of the hormetic concept was a discovery by Prof. Hugo Schulz, that bacterial metabolism was stimulated. However, it might be important to distinguish between increased metabolism that is beneficial for the cell and increased metabolism that has detrimental effects. With that in mind, the increase in metabolic activity at higher concentrations does not necessarily mean an increase in proliferation. If a cell shifts its metabolism and increases flux through pathways that yield reducing agents then the response to the MTT reagent is heightened. Therefore, the previously made assumption (more metabolic activity equals more metabolically active cells) would not apply here and it would be important to investigate which pathways are induced clarify the situation.

In general, it is known that resveratrol influences and mimics energy restriction through the interaction with AMPK and SIRT1,[88,108,109] and triggers apoptosis through increased p53 expression, caspase 9 and 3 activation, and a shift in Bax/Bcl-2 ratio.[88,90] Long-term exposure studies on mesenchymal stem cell revealed hormetic behavior in the concentrations range from 0.1 to 5 µM during a 30 day period,[110] hepatic stellate cells (GRX) did not experience hormesis after 24 or 120 hours of exposure, just a decrease in cell viability.[111] These two examples show the importance of a completely resolved concentration and duration profile, especially in differing cell lines.

3.3. Conclusion

Four compounds were investigated for their hormetic behavior in a human hepatocellular cancer model in terms of metabolic activity and plasma membrane degradation. Epigallocatechin gallate, quercetin, and curcumin did not show significant hormetic behavior. Resveratrol exerted hormetic behavior in a time- and dose-dependent manner associated with concentrations of 50, 100 and 250 µM. Low concentrations up to 10 µM showed a trend toward hormesis, albeit not statistically significant, which was opposite to the trend observed for higher concentrations. Cellular behavior associated with 100 µM resveratrol deserves a short note, since both, metabolic activity and plasma membrane degradation showed higher values. This points to increased signaling and reductive species production associated with pathways that eventually affect plasma membrane integrity. The herein presented data therefore adds to the literature that supports resveratrol as a hormetic compound.

Vargas and Burd (2010) bring up an important point in their review: Most studies on hormetic compounds only involve *in vitro* and rarely *in vivo* work. [3] From *in vitro* and *in vivo* work, it is almost too farfetched to give recommendations on supplemental ingestions of specific phytochemicals. Conclusion like these would require a lot more extensive basic research into the biochemical processes associated with each phytochemical. One needs to account for clearing rates in a cell, residence times in a cell, metabolism until the compound reaches the target cell, the ultimate concentration inside the cell, and the individual variability in metabolism rate. Especially since, if a compound exhibits hormetic features, a low concentration (aka not enough of the phytochemical ingested) can have the exact opposite outcome than the desired one. This concern

has already been voiced by Calabrese *et al.* (2010): with respect to anticarcinogenic characteristics, a low dose increases instead of decreases the risk of tumor cell growth.[89] Hormesis is undoubtedly a phenomenon that requires more in-depth attention and better method design. The two main questions, if there is a universal underlying pathway for hormesis, or if a compound triggers its own profile of distinct beneficial processes inside the cell, have not been answered yet, despite the growing literature on hormesis. The example of resveratrol, however, shows how important it is to incorporate a wide range of concentrations and include several more time points (exceeding the norm of 24 hours).

4. Future Directions

Resveratrol causes a noticeable switch in cellular behavior in HepG2 cells between 12 and 24 hours of exposure, significantly different especially at concentrations from 50 to 250 μM. These findings open the floor to studies that further explore the metabolic basis of hormesis associated with resveratrol. Future experiments should include the assignment of exact post exposure timepoints with real-time assays, to pinpoint when signaling in the cell changes, and consecutive analysis of metabolic markers at these times.

4.1. Assigning Crucial Energetic Signaling Timepoints

Since virtually every metabolic pathway requires energy, changes in cellular energy production, and changes in cellular ATP, can be a clue for an adaptive change. Luciferase assays have been used to determine the concentration of ATP in cell lysate and to verify cell viability in endpoint assays,[94] however, an experiment aiming to find specific time points would have to be a real-time assay. Vrana *et. al* (2014) used sets of simple cell-based real-time assays in combination with mathematical loops (accounting for aerobic and anaerobic energy metabolism) to estimate the relative intracellular ATP concentration and therefore define the cell's metabolic energy state.[112] Extracellular oxygen consumption and intracellular NADH were measured kinetically for 24 hours and the data fed into the following expression to calculate theoretically available energetic phosphate:

$$If\ 6[O_2]_{sample} - 2\,[NADH]_{sample} > 0$$

$$Then\ PO_4 = \frac{6\,[O_2]_{sample} - 2[NADH]_{sample}}{4}$$

$$\text{Else } \mathrm{PO_4} = \frac{[\mathrm{NADH}]_{\mathrm{sample}}}{4}$$

In theory, the concept can be expanded to eventually calculate the actual concentration of ATP per cell, using two cell based assays, one to monitor cell density in real time (for example RealTime-Glo™), and another one to measure ATP at distinct endpoints (for example CellTiter-Glo® 2.0).

Both of these assays are based on a bioluminescent signal, which provides the option to run experiments in white or black (if multiplexed with fluorescence based assays) 96 well plates and conveniently obtain data from a plate reader or luminometer.

RealTime-Glo™, for example, has been used to validate cellular health in tumor resistance experiments,[113] investigate the cytotoxicity of bacterial biofilms from wound dressings,[114] or study glucose uptake in a multiplexed assay.[115] The basis of the kit is the conversion of a luciferase prosubstrate (identity proprietary to Promega) into an active luciferase substrate if taken up into a viable cell. The reducing environment inside the cell facilitates conversion, the substrate exits the cell into the medium where it causes luminescence through the interaction with luciferase.[116] This way, the signal correlates with active metabolism inside a cell, or put differently, with the production of reducing molecules, such as NADH or FAD, and the manufacturers claim a linear increase in signal with cell density.[117] Since the substrate is used up by the luciferase, the signal does not compound over time, which increases the accuracy of real-time measurements and provides a big advantage over conventional cell viability assays, such as Calcein AM, in addition to the nontoxic nature of the RealTime-Glo™ reagents. With this assay, it would be possible to determine the cell number over a given exposure time, granted a standard curve of known densities would be prepared and measured right before the kinetic measurement, ideally in the same plate. Then, available energetic phosphate/cell could be estimated:

$$\text{available } PO_4 = \frac{PO_4}{\text{cell density}}$$

The second assay, in varying version of the product, has been employed by several studies to measure cell viability.[84,113,118–120] It can also be used to estimate cell density,[121] or just simply measure the content of ATP itself,[122] since the assay is based on the reaction of ATP with the substrate luciferin, which results in a bioluminescent signal directly correlated with the concentration of the energy carrier inside a cell. This can be used to compare the estimated available energetic phosphate, to the actual available triphosphate nucleotide. CellTiter-Glo® 2.0 features a solution-mix that contains cell lysis buffer and luciferin as assay reagent. With this solution, the separate cell lysis step and the consecutive centrifugation to obtain ATP in just the lysate is eliminated and the relative amount of ATP can be directly measured in the 96 well reaction plate. A calibration curve of known amounts of analyte can add the important quantitative aspect.

Since the assay is destructive and only allows for endpoint measurements, specific time points (such as six, twelve, and 24 hours for a 24-hour kinetic experiment) should be chosen to measure definite amounts of adenosine triphosphate. The real ATP can then be compared with the estimated concentration at the same time point, a correction factor can be calculated, and finally it should be possible to convert the entire 24 hour estimated data into real ATP/cell. Graphic visualization in a contour plot heat map and calculation of bifurcation points[112] can aid in the identification of critical time points of changes in energetic metabolism.

4.2. Resolve Metabolic Switches in Cellular Energy Metabolism

To understand the fundamental events that change a cell's behavior after exposure to a xenobiotic, several questions need to be addressed: If the cell increases ATP production, which pathways are increased in activity? Are other pathways decreased in activity? If the cell decreases ATP production, is it bound to die shortly after or does it stay in a lower energetic state and survives? With respect to resveratrol and its hormetic character, two cellular mechanism are of interest especially when it comes to hormesis: autophagy and apoptosis.

Once critical time points are identified, experiments should focus on endpoint assays at these times points to study changes in certain signaling markers. As it is still unclear if common pathways cause hormetic behavior, such as apoptosis and autophagy, or if a compound triggers its own profile of distinct beneficial processes inside the cell, several factors could and should be studied. Kits that label initiator and effector caspases are widely available, as well as antibodies to quantify Atg, which should give a good understanding of the downstream effects for the pathways of autophagy and apoptosis. Additionally, signaling associated with nutrient sensing molecules, such as mTOR, AMPK, Ca^{2+} and SIRT1 can provide an insight into the metabolic side, whether metabolism shifts towards catabolic, energy-yielding processes or conserves energy, for example. The change in redox environment inside the cell would be a useful indicator to measure and would most likely support the theory that hormesis is a phenomenon based on compound-specific cellular effects.

Besides expanding the in-depth study of resveratrol, the general experimental concepts can easily be adapted to other substances, such as sulforaphane (see Appendix A).

Appendix A - Hormetic Behavior of Sulforaphane

In addition to the compounds covered in the main text, sulforaphane was also screened for its hormetic behavior in the same cell model (HepG2).

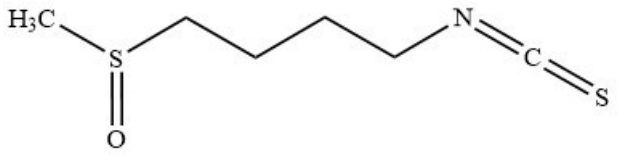


Figure A-1| Chemical Structure: Sulforaphane.

Over the past few years, sulforaphane has received a decent amount of scientific attention. The compound is an isothiocyanate and a product of an enzymatic hydrolysis of glucoraphanin, a substance commonly found in cruciform vegetables, the most recognized being broccoli.[123–125] The enzyme (myrosinase) responsible for the release of sulforaphane is found right in the vegetable itself, but can also be found in human colon microbiota. Among a plethora of beneficial effects, it has been found to increase cell proliferation and angiogenesis,[126] for example, and to have antioxidant and anti-inflammatory traits while displaying the characteristic biphasic effects of a hormetin: Cytoprotective and proliferative effects of sulforaphane have been ascribed to concentration levels below 10 µM for several cell lines, including HepG2;[118,126] higher levels though appear to decrease cell proliferation and induce apoptosis.[118,127] Park *et al.* (2007) found an increase in pro-apoptotic Bax expression and a simultaneous decrease in Bcl-2 and Bcl-X_L expression (anti-apoptotic factors) after HepG2 cells were exposed to sulforaphane,[127] while Zou *et al.* (2017) attribute the onset of apoptosis to endoplasmic reticulum stress as they observed increased expression of Bip, Bid, XBP-1, CHOP, as well as caspase-12.[128] The compounds antioxidant effect stems from the release of nuclear factor erythroid 2-related factor 2 (Nrf2) from the Keap1/Cul3/Rbx1/E3 complex* upon binding of sulforaphane.[123,126] Liberated Nrf2 induces expression of superoxide dismutase 1 (SOD1) and catalase[129] in the nucleus, two major antioxidant enzymes. A wide range of scientific publication suggests that sulforaphane also increases phase II detoxification, especially with respect to carcinogens,[130] through an increase in glutathione

* A complex formed by the noncovalent association of these four peptides: Kelch-like ECH-associated protein 1 (Keap1), Cullin 3 (Cul3), ring box 1 (Rbx1), and ubiquitin ligase E3 (E3). It serves as a redox sensing formation in the cytosol that binds Nrf2 under low oxidative cellular conditions.[123]

S - transferase (GST), glutathione levels in general, thioredoxins, as well as quinone reductase,[118,130–132] and reduces phase I metabolism,[123,133] which can decrease a xenobiotics harmful potential.

Effects on Metabolic Activity

Materials and Methods

HepG2 cells were exposed to sulforaphane in varying concentrations (0.01 – 500 µM) for 24 hours. Sulforaphane was acquired from Cayman Chemical Company. HepG2 (human hepatocellular carcinoma-derived) cells were cultured in Gibco® RPMI-1640 Medium without L-glutamine, supplemented with 5% FBS, 2% penicillin and streptomycin, and 1% L-glutamine, and maintained at 37 °C in humidified, 5% CO_2 atmosphere. Before lifting, cells were washed twice with cold phosphate buffered saline. A stock solution of 50 mM was prepared in DMSO. 100X dosing solutions were freshly mixed by serial dilution of the stock solution in DMSO. These were subsequently diluted 100-fold in culture media. The dosing solutions had a final DMSO concentration of 1%. Cells were plated in a clear 96 well plate at a density of $60x10^3$ cells/well in 100 µL media and allowed to reattach for 24 hours. At this point, spent media was removed and 100 uL fresh media supplied, which contained the respective concentration of sulforaphane.

The MTT reagent was prepared by dissolving 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide in PBS to a concentration of 5 mg/mL. The detergent was a solution of 10% SDS in DMSO containing 0.6% glacial acetic acid. Briefly, after 24-hour exposure, 20 µL of MTT reagent were added to each well. After 6 hours of incubation at 37 °C, 100 µL/well were dispensed and the plates allowed to rest for 5 minutes before they were shaken on an orbital shaker at 500 rpm for 5 minutes. Absorbance was measured immediately after at 570 nm wavelength on a Fluostar Optima (BGM Labtech, Germany). Controls for this assay included untreated 1% DMSO vehicle controls, as well as blank controls without cells at each concentration for background absorbance, since sulforaphane showed an increased absorbance with increased concentration, especially upon addition of the MTT reagent.

Results and Conclusion

After 24 hours of exposure to sulforaphane (0.01 – 500 µM), a significant increase in metabolic activity is seen at 5 µM and 10 µM compared to the untreated control (Figure A-1, Table A-1). A slight increase is noticeable, although not statistically significant, starting from 0.1 µM and steadily rising to 5 µM. By that point, activity had increased by 53% in the mean. After that, the effect receded: 10 µM still result in a 38% higher-than-control activity, 25 µM tends to be less than 20% higher. The response to 50 µM changed significantly compared to 25 µM, where metabolic activity already decreased down to 70% of the control value. Half of the metabolic activity was lost at 70 to 100 µM. Virtually no metabolic activity (3%) was observed when cells were treated with 200 µM of sulforaphane, or more.

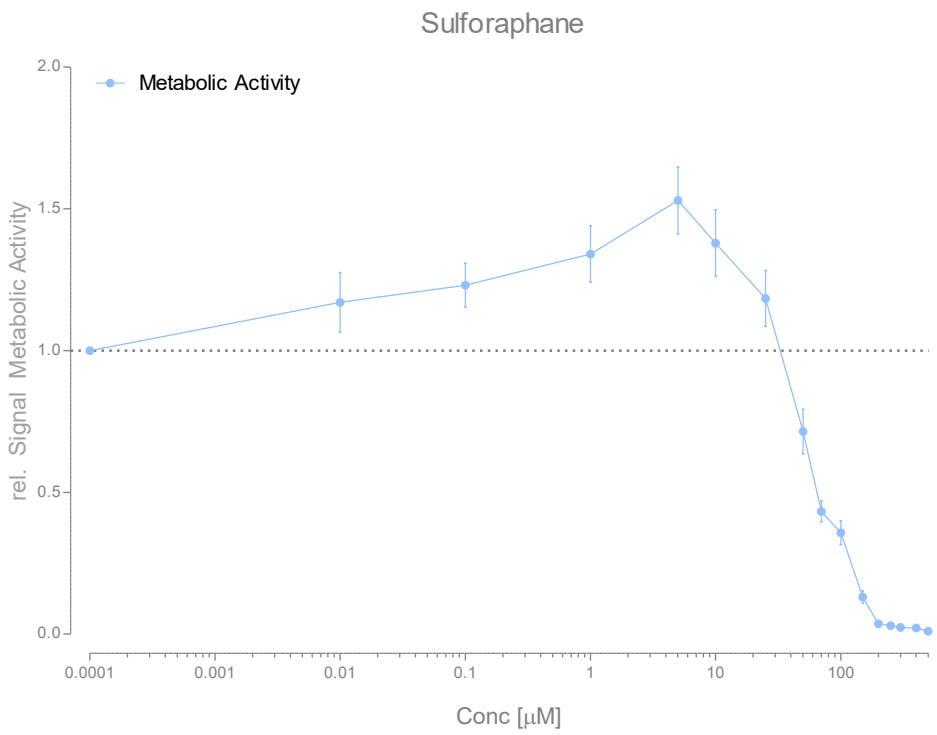


Figure A-2| Metabolic activity after 24-hour exposure to sulforaphane.

HepG2 cells were exposed to varying concentrations of sulforaphane for 24 hours. Effects on metabolic activity were assessed using MTT. Data shown represent the mean and the standard error of the mean from six independent experiments. Experimental values were measured in triplicate.

Table A-1| Summary of statistically significant changes in metabolic activity by sulforaphane concentration compared to untreated vehicle controls and previous concentrations.

	Compared to Untreated Vehicle Control		Compared to Previous Concentration	
	Concentration [µM]	* p≤0.05 ** p≤0.01 *** p≤0.001	Concentration [µM]	# p≤0.05 ## p≤0.01 ### p≤0.001
Metabolic Activity	5	***	50	###
	10	*		
	70 - 500	***		

These results confirm previously reported observations[119,128,130,134] and recommend sulforaphane as a prime subject for further investigation in plasma membrane integrity measurements and real-time experiments. However, data shown and discussed in Appendix B draws doubt on the just presented data and the reliability of the performed experiments under the chosen experimental conditions.

Appendix B - Impact of Cell Population and Instrumentation

The data presented in the main text was uniformly acquired in the same laboratory, using the same cell culture reagents, the same equipment, and instrumentation. However, comparative data for epigallocatechin gallate, quercetin, and resveratrol was also acquired in a laboratory at the Fulda University of Applied Sciences with a different population of HepG2 cells, different reagents, and instrumentation. Instrument bias and speculations on the source of cell population variability have been discussed in literature before,[135–137] the following is a comparison of the data gathered in both laboratories, which impressively shows the impact of cell population and instrumentation on research outcomes.

Methods and Materials

Materials and methodology are described in detail in the main text for the data labeled "West Virginia University". Data labeled with "Fulda U.o.A.Science" was acquired as presented below:

Materials

All materials were acquired from VWR, unless otherwise specified. Quercetin, (-)-epigallocatechin gallate, and trans-resveratrol, were obtained from Cayman Chemical Company.

Cell Culture

HepG2 (human hepatocellular carcinoma-derived) cells were cultured in Gibco® RPMI-1640 Medium without L-glutamine, supplemented with 5% FBS, 2% penicillin and streptomycin, and 1% L-glutamine, and maintained at 37 °C in humidified, 5% CO_2 atmosphere. Before lifting, cells were washed twice with cold phosphate buffered saline.

MTT Assay

Cell viability assays were performed using these solutions: The MTT reagent was prepared by dissolving 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide in PBS to a concentration of 5 mg/mL. The detergent was a solution of 10% SDS in DMSO containing 0.6% concentrated acetic acid. Briefly, after 24-hour exposure, 20 µL of MTT reagent were added to

each well. After 6 hours of incubation at 37 °C, 100 µL/well were dispensed and the plates allowed to rest for 5 minutes before they were shaken on an orbital shaker at 500 rpm for 5 minutes. Absorbance was measured immediately after at 570 nm wavelength on a Fluostar Optima (BGM Labtech, Germany). All other aspects were similar to the procedures laid out in the main text.

Results and Discussion

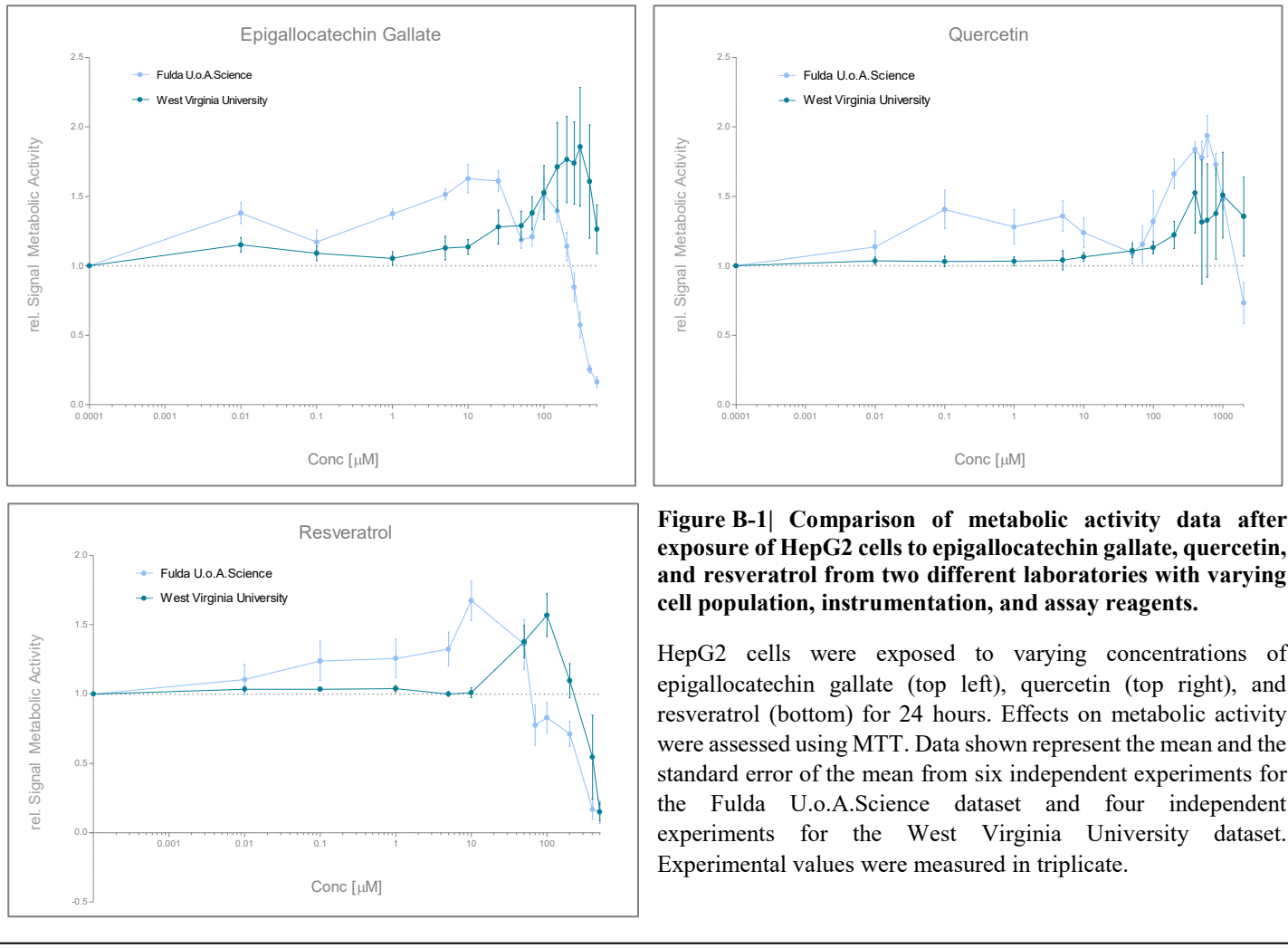


Figure B-1| Comparison of metabolic activity data after exposure of HepG2 cells to epigallocatechin gallate, quercetin, and resveratrol from two different laboratories with varying cell population, instrumentation, and assay reagents.

HepG2 cells were exposed to varying concentrations of epigallocatechin gallate (top left), quercetin (top right), and resveratrol (bottom) for 24 hours. Effects on metabolic activity were assessed using MTT. Data shown represent the mean and the standard error of the mean from six independent experiments for the Fulda U.o.A.Science dataset and four independent experiments for the West Virginia University dataset. Experimental values were measured in triplicate.

Table B-1| Comparative summary of statistically significant changes in metabolic activity by compound concentration compared to untreated vehicle controls and previous concentrations.

	Compared to Untreated Vehicle Control		Compared to Previous Concentration	
Metabolic Activity	Concentration [µM]	* p≤0.05 ** p≤0.01 *** p≤0.001	Concentration [µM]	# p≤0.05 ## p≤0.01 ### p≤0.001
Epigallocatechin Gallate				
West Virginia University	No statistically significant changes		No statistically significant changes	
Fulda U.o.A.Science	5	**	50	#
	10 & 25	***		
	100	**		
	300	*		
	400 & 500	***		
Quercetin				
West Virginia University	No statistically significant changes		No statistically significant changes	
Fulda U.o.A.Science	200 & 400	*	2000	##
	500	**		
	600	***		
	800	**		
Resveratrol				
West Virginia University	500	***	No statistically significant changes	
Fulda U.o.A.Science	10 & 400	**	No statistically significant changes	
	500	***		

Metabolic activity in response to a 24-hour exposure of three compounds (epigallocatechin gallate, quercetin, and resveratrol) was investigated in two laboratories, one at West Virginia University, the other one at Fulda University of Applied Science (Fulda U.o.A.Science). Cell culture stock and reagents, assay conditions, and instrumentation varied between the two laboratories, which was clearly observable in the acquired data.

In the case of epigallocatechin gallate, the mean values from Fulda describe a very interesting behavior: Metabolic activity did not change significantly up to 5 µM compared to vehicle control, but at 5 µM activity increased by 51% in the mean and even by 63% and 61% at 10 µM and 25 µM, respectively. 50 µM did not change the response significantly compared to vehicle controls, but compared to the previous concentration of 25 µM the signal decreased markedly. 100 µM again boosted metabolic activity 53% above the control level. Concentrations higher than 100 µM then continuously impeded metabolic response, for example down to about 50% of the control value from 300 µM exposure and less than 20% for the highest exposure of 500 µM.

The cellular response was not reproducible in the American laboratory. High variability in the independent experiments drove the standard error of the mean, therefore no statistically significant changes in metabolic activity were found. However, a slight trend of increased metabolic activity past 100 µM was observed, although the signal did not decrease below control values even at the highest dose.

Similarly, trends from resveratrol exposure shifted and were not reproducible. While the data from Fulda point to a statistically significant peak increase in metabolic activity at 10 µM (67%) and a decrease of activity starting at 70 µM, data from West Virginia University does not show a significantly increased response until 50 µM and 100 µM (38% and 57%, respectively) and a decreased activity starting between 200 and 400 µM. Only 50 µM and 500 µM evoked a comparable cellular metabolic activity.

The shift in concentration response was somewhat less pronounced when cells were exposed to Quercetin. Concentrations between 200 µM and 1000 µM increased the metabolic activity by 50% to almost 100% of the vehicle control values. Lower concentrations in the range of 0.1 µM to 10 µM also strengthened metabolism noticeably up to 40%, but not statistically significantly. Compared to that, no statistically significant changes were observed in the experiments from West Virginia University, especially at higher concentrations, due to exceptionally large standard errors. A small trend of increased metabolic activity could be inferred at 200 µM. Smaller doses from 0.01 µM to 10 µM demonstrated no noticeable difference.

The data provided is a prime example of the impact of instrumentation and reagent/procedure consistency on experimental outcome. There is no method to determine which laboratory results are closest to the actual cellular behavior, unless more independent trials are performed. Even then it is not guaranteed that the results will converge, as the genetic and behavioral variation between separate populations of the same cell line can be, as demonstrated, quite large. It would be interesting to see how large the difference in results would be with the same protocols, chemicals, and instrumentation to assess the true impact of cell population. Single cell measurements might be a better way to study the exact effects of hormetic compounds, since it has been shown before that actual signaling events can be significantly different from what cell population studies suggest.[12]

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