MECHANISTIC NETWORK MODELS IN SAFETY AND TOXICITY EVALUATION OF NUTRACEUTICALS

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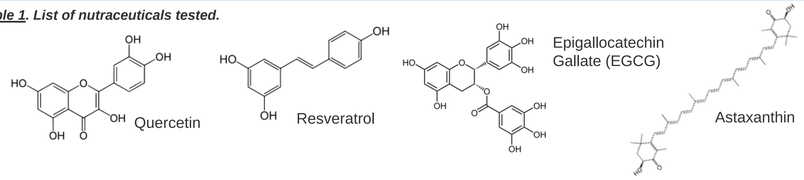
INTRODUCTION

Nutraceuticals are food constituents with potential health benefits beyond their nutritional value. *In vitro* and *in vivo* studies suggest a protective effect of nutraceuticals against chronic diseases, but the results are not fully supported by clinical evidence. Nutraceuticals are generally recognized as safe at dietary doses; however, they are often consumed at higher doses in the form of nutritional supplements. Exposure to supra-dietary doses of nutraceuticals is of toxicological concern, particularly because some substances may cause genomic changes in target tissues.

Here, we assessed the effect of four nutraceuticals, resveratrol (a polyphenol present in grapes and berries), quercetin (a flavonol found in many fruits, vegetables, leaves and grains), astaxanthin (a keto-carotenoid found in microalgae, yeast, many fishes and crustaceans), and epigallocatechin gallate (a polyphenol present in tea and in various vegetables), in two human primary cell types, hepatocytes and coronary artery endothelial cells (HCAECs), using a multi-step systems biology approach combining (STEP 1) real-time cell viability measurements with (STEP 2) a panel of high-content screening (HCS) endpoints and (STEP 3) gene expression changes analysis based on computable biological network models.

MATERIALS AND METHODS

***Table 1**. List of nutraceuticals tested.*



Toxicological assessment of the above nutraceuticals was done on human primary hepatocytes and HCAECs using the following multiple-step approach:

1. Cell viability was measured using a real-time analyzer during the exposure to a large concentration range of each nutraceutical (STEP 1)
2. Concentration- and time-dependent effects were assessed using a panel of endpoints measured using HCS technology ***[1]*** (STEP 2)
3. Transcriptomics data wee generated from cells exposed to nutraceutical concentrations selected based on the HCS results (concentrations inducing less than 30% cell count decrease) and exposed for 24h or 72h. Network based approach is applied to analyze and quantify the biological perturbations induced by nutraceutical exposure (STEP 3)

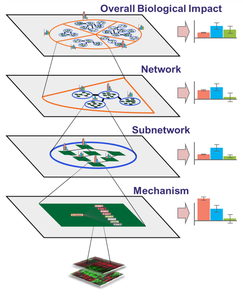
STEP 1 – Real-time cell analyzer

STEP 2 – High-Content Screening (HCS)

***Table 1**. List of HCS–based endpoints*

• Cell count	• Stress kinase (p-cJun)	• Mitochondrial mass
• Nuclear size	• Oxidative stress (ROS)	• Apoptosis (Caspase 3/7)
• DNA structure	• Glutathione Content	• Cytochrome C release
• DNA damage (p-H2AX)	• Mitochondrial potential	• Cell membrane permeability
	• Steatosis (Hepatocytes)	• Phospholipidosis (Hepatocytes)

STEP 3 – Transcriptomics analysis



Overall Biological Impact Factor (BIF) is computed as a linear combination of network scores

Network activity score is computed as a linear combination of the scores of subnetworks within the network

Subnetwork activity score is computed as a linear combination of the scores of mechanisms within the subnetwork

Mechanism activity score is computed as a linear combination of the differential expression of genes supporting the mechanism

Figure 1. The causal biological networks are describing biological processes or mechanisms (e.g., Cell Proliferation [2], Cell Stress [3], DNA damage and Apoptosis [4] or Inflammation [5]). They are composed of backbone nodes connected by causal directional relationships (= edges) derived from an evidence line extracted from literature. Differential expression of genes are experimental evidences for the activation of upstream backbone node. Differentially expressed genes are translated into Network Perturbation Amplitude (NPA) scores [6] for each biological networks and sub-networks allowing a higher granularity of the biological interpretation of the transcriptomic dataset. The Biological Impact Factor (BIF) [7] is computed by aggregating NPA scores. It represents a hollistic score that describes the system-wide effect of all biological processes perturbed after exposure.

RESULTS

HEPATOCYTES

Nutraceuticals effect on hepatocyte viability and steatosis/phospholipidosis after 72h exposure.

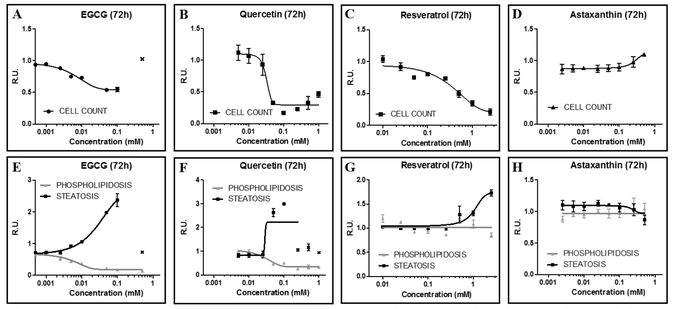


Figure 2. After 72h exposure to nutraceuticals (ECGC, quercetin, resveratrol, and astaxanthin), cell viability (A-D) as well as phospholipidosis and steatosis related endpoints were measured in hepatocytes. Values are normalized to vehicle controls and represent mean ± SD from three biological replicates in one experiment. X indicates values excluded from curve fitting.

Overall Biological Impact Factor measured in hepatocytes exposed for 24h and 72h to two concentrations of nutraceuticals.

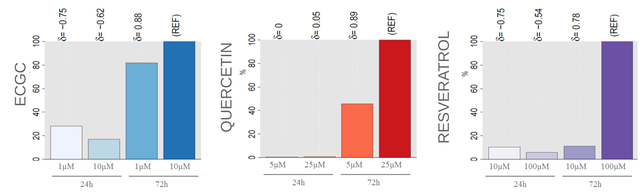


Figure 3. The percentages on the y axis give the relative biological impact that was derived from the cumulated network perturbations caused by the treatments relative to the reference (REF) (defined as the treatment comparison with the highest perturbation). For each treatment comparison, the δ value (−1 to 1) indicates how similar the underlying network perturbations were with respect to the reference. A value of 1 indicates that all the networks are perturbed by the same mechanisms.

Evaluation of individual contributions to overall Biological Impact Factor values for hepatocytes exposed to two concentrations of EGCG, quercetin, or resveratrol for 24h and 72h.

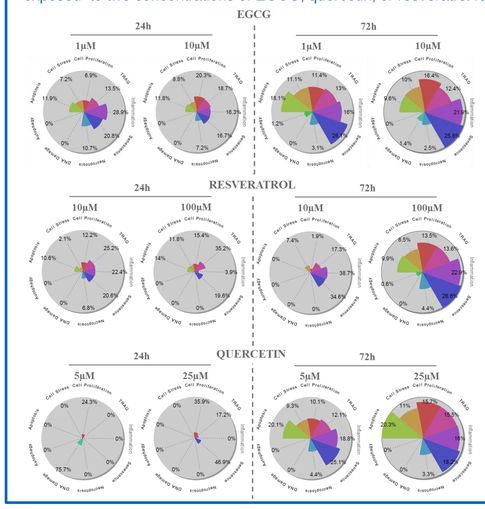


Figure 4. The star plots illustrate the decomposition of the overall relative BIF value into the different network components for each treatment group (per concentration and per exposure time). The surface area of each slice is proportional to the contribution of each network perturbation (shown as percent in the labels) for a particular nutraceutical. It is further adjusted by the relative BIF for the treatment compared with the reference so that the sum of the slice areas for each treatment equals the BIF for the treatment. The pie chart represents the distribution of the sum of contributions for each network across all treatment groups; they all sum to 100%. TRAG, tissue repair and angiogenesis network.

HCAECs

Nutraceuticals effects on HCAEC viability after 4h and 24h of exposure.

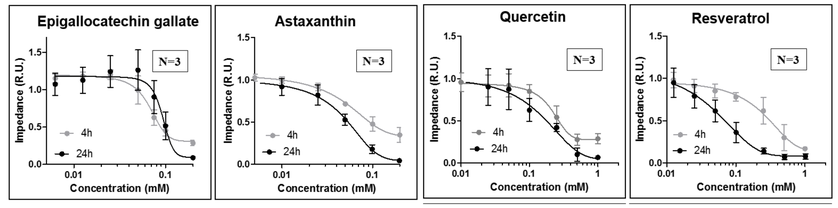


Figure 5. Exposure to each nutraceutical caused a concentration-dependent decrease in HCAEC viability at both time points. Values were normalized to the vehicle control. Values represent mean ± SEM from three independent experiments.

HCS analysis of nutraceuticals effects on HCAECs after 4h and 24h of exposure.

NUTRACEUTICALS (HCAEC)		Cell Loss	DNA Damage	Stress Kinase	GSH Content	Oxidative Stress	Caspase 3/7	Cytochrome C Release	Cell Membrane Permeability	Mitochondrial Membrane Potential	Mitochondrial Mass
QUERCETIN	4h	-	-	-	✓✓ (100μM)	✓✓ (250μM)	✓✓ (500μM)	-	✓✓ (500μM)	✓ (250μM)	✓✓ (250μM)
	24h	✓✓ (250μM)	✓✓ (100μM)	✓✓ (100μM)	-	✓✓ (250μM)	✓✓ (250μM)	-	✓✓ (500μM)	✓ (250μM)	-
EGCG	4h	-	✓✓ (75μM)	-	✓✓ (100μM)	-	-	-	-	✓✓ (100μM)	✓ (100μM)
	24h	✓✓ (100μM)	✓✓ (75μM)	✓ (75μM)	✓✓* (100μM)	-	✓✓ (50μM)	-	✓ (75μM)	✓✓ (100μM)	-
ASTAXANTHIN	4h	✓ (200μM)	-	✓ (200μM)	✓✓ (200μM)	✓✓ (100μM)	-	✓ (200μM)	-	-	-
	24h	✓✓ (100μM)	✓ (200μM)	✓✓ (100μM)	✓✓* (50μM)	✓✓ (50μM)	✓✓ (100μM)	✓✓ (100μM)	-	-	-

Table 2. Results were considered positive (✓✓) if a concentration-dependent response was observed with at least a 2-fold increase in signal over the vehicle control (or a 50% decrease in signal in the case of cell count and GSH content). Signal increases between 1.5- and 2-fold (or a decreases between 30% and 50% in the case of cell count and GSH content) were considered weakly positive (✓). Data represent results from at least three independent experiments.* indicates an increase in GSH levels.

CONCLUSIONS

Our results showed that EGCG, astaxanthin, resveratrol, and quercetin are toxic to primary human hepatocytes and/or endothelial cells at micromolar concentrations. Moreover, we observed some similarities between the compounds, notably in terms of induction of oxidative stress and cell stress responses. This is an interesting result because the claimed potential health benefits of these nutraceuticals are based largely on their reported anti-oxidant properties. However, our results are more in line with previous reports indicating that these compounds can also show pro-oxidant activity under certain conditions. While micromolar concentrations are high compared with typical dietary exposures, it cannot be ignored that continuous use of enriched nutritional supplements could result in systemic concentrations compatible with these reported effects.

REFERENCES

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