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Network pharmacology of ginseng (Part II): The differential effects of Red Ginseng and ginsenoside Rg5 in cancer and heart diseases as determined by transcriptomics

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Abstract: *Panax ginseng* C.A.Mey. is an adaptogenic plant traditionally used to enhance mental and physical capacities in cases of weakness, exhaustion, tiredness, loss of concentration, and during recovery. According to ancient records, Red Ginseng root preparations enhance longevity with long-term intake. Recent pharmacokinetic studies of ginsenosides in humans and our *in vitro* study in neuronal cells suggest that ginsenosides are effective when their level in blood is shallow - at concentrations from 10^{-6} to 10^{-18} M. In the present study, we compared the effects of Red Ginseng root preparation HRG80™(HRG) at concentrations from 0.01 to 10,000 ng/ml with effects of White Ginseng (WG) and purified ginsenosides Rb1, Rg3, Rg5 and Rk1 on gene expression of isolated hippocampal neurons. The aim of this study was to predict the effects of differently expressed genes on cellular and physiological functions in organismal disorders and diseases. Gene expression profiling was performed by transcriptome-wide mRNA microarray analyses in murine HT22 cells after treatment with ginseng preparations. Ingenuity pathway downstream/upstream analysis (IPA) was performed with datasets of significantly up-or downregulated genes, and expected effects on cellular function and disease were identified by IPA software. Ginsenosides Rb1, Rg3, Rg5, and Rk1 have substantially various effects on gene expression profiles (signatures) and are different from signatures of HRG and WG. Furthermore, the signature of HRG is changed significantly with dilution from 10000 to 0.01 ng/ml. Network pharmacological analyses of gene expression profiles showed that HRG exhibits predictable positive effects in neuroinflammation, senescence, apoptosis, and immune response, suggesting beneficial soft-acting effects in cancer, gastrointestinal, and endocrine systems diseases and disorders in a wide range of low concentrations in blood.

Keywords: Red Ginseng HRG80™, Ginsenoside Rg5, Gene expression, IPA pathways, Network pharmacology, Transcriptomics

1. Introduction

Panax ginseng C.A.Mey. is likely one of the most widely used botanicals in the world [1,2]. This adaptogenic plant [2-8] is approved in Europe and other countries as an herbal medicinal product to enhance cognitive functions, physical capacities in weakness, exhaustion, tiredness, loss of concentration, and during convalescence [9,10]. According to ancient records, Red Ginseng root enhances longevity with long-term intake [11]. Overall, ginseng is a promising treatment for aging-related diseases, including neurodegenerative, cardiovascular diseases, diabetes, and cancer [1-15].

A challenge in ginseng research and herbal therapy, in general, is the consistency and reproducibility of the results obtained from various studies [10]. One of the critical solutions to this problem is the reproducible quality of herbal preparations, which is possible to achieve by implementation GMP and Good Agriculture Practices in the production of herbal preparations, including their cultivation in well-controlled conditions.

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Recently, we have demonstrated that both hydroponically cultivated Red Ginseng root powder HRG80™ (HRG) and White Ginseng (WG) preparations were effective in preventing and mitigating the stress-induced deterioration of cognitive functions in healthy subjects [16] and elderly patients with mild cognitive disorders [17] at daily doses of 418 mg HRG (64 mg of total ginsenosides, 1.2 mg Rg5) and 764 mg WG (19.6 mg of total ginsenosides, 0.16 mg Rg5). Both HRG and WG preparations substantially impacted brain activity, affecting various brain regions depending on the mental load during relaxation and cognitive tasks associated with memory, attention, and mental performance. Both ginseng preparations activated electroencephalogram (EEG) spectral powers compared to placebo [17]. The spectral changes in the quantitative EEG induced by HRG indicated an improvement in mood and calming effects evidenced by the modulation of β2 waves, representing changes in GABA-ergic neurotransmission. HRG attenuated δ/θ waves power, which is increasing with aging [17]. Red Ginseng preparation HRG was more active than WG in humans [17] and an animal study, where HRG induced higher excitability of pyramidal cells by modulation of ionotropic glutamate NMDA and Kainate receptor-mediated transmission at daily doses of 10 to 50 mg/kg body weight corresponding to human doses of 100-500 mg [18].

We had several aims in this study, intending to cover several gaps in ginseng research and specifically to hydroponically cultivated roots used to produce red ginseng preparation. They are related to the following issues.

Numerous studies of purified ginsenosides show their potential efficacy in treating cancer and other diseases [19-28], suggesting that the total extract or powdered roots have similar or probably better therapeutic effectiveness [23-26]. However, the clinical evidence supporting this suggestion in comparative studies is missing [27,28].

As a matter of fact, the blood cells and tissues are continuously exposed to varying concentrations of ginsenosides after oral administration of ginseng in the daily therapeutic doses of 0.6-9.0 g [10,29,30]. They range from maximal detected concentrations of 35 ng/ml to the limit of detection 0.5 ng/ml and less within 12-48 h after oral intake [29]. A recent study demonstrated that ginsenoside Rg5 exhibits soft-acting effects in a wide range of physiological and sub-physiological concentrations from 1 μM to 1 aM. However, low-dose studies in corresponding concentrations are not available in scientific literature.

Pharmacological effects of ginseng preparations at various levels of regulation of homeostasis have been studied [26, 31-38]; however, network pharmacology studies [26, 33-38] at the transcriptomic level of regulation of cellular homeostasis are limited [38].

Therefore, the first aim of this study was to compare the effects of Red Ginseng root preparation (HRG80 ™) at concentrations from 0.01 to 10,000 ng/ml with effects of White Ginseng (WG) and purified ginsenosides Rb1, Rg3, Rg5, and Rk1 on gene expression of isolated hippocampal neurons. The study's second aim was to predict the effects of differently expressed genes on cellular and physiological functions in organismal disorders and diseases using a network pharmacology approach to transcriptomics.

2. Results

2.1. *Effect of ginsenosides Rb1, Rg3, Rg5 and Rk1, WG and HRG80™ at different concentrations on gene expression profile in the hippocampal neuronal cell line HT22.*

Table 1 shows the number of genes deregulated (>20 fold compared to control) by ginsenosides Rb1, Rg3, Rg5 and Rk1, WG and HRG80 ™ at different concentrations in the hippocampal neuronal cell line HT22, which in the same range – from 283 to 461 at all tested concentrations of HRG80 ™ and within 20% RSD of the mean value 383 ± 77 (**Supplemental Table S1** in **Supplement 1**).

The number of genes deregulated by WG and purified ginsenosides is also within this range, despite the difference in the content of ginsenosides Rb1, Rg3, Rg5, and Rk1 in WG and HRG80 ™ (**Table 1**). However, the gene expression profile (signature) was substance-specific and concentration-specific, containing many genes that are not deregulated in other concentrations or other test samples alone (**Figure 1**, **Table 1**, and **Supplemental Table S2** in **Supplement 2**).

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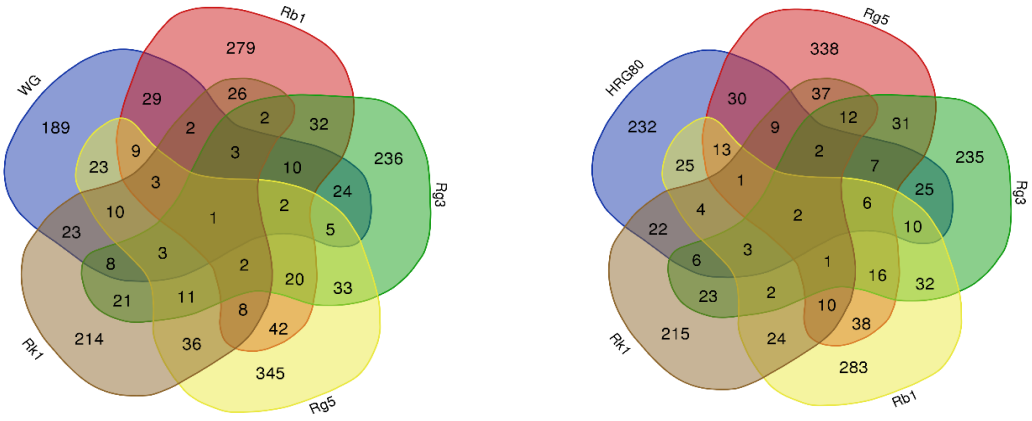
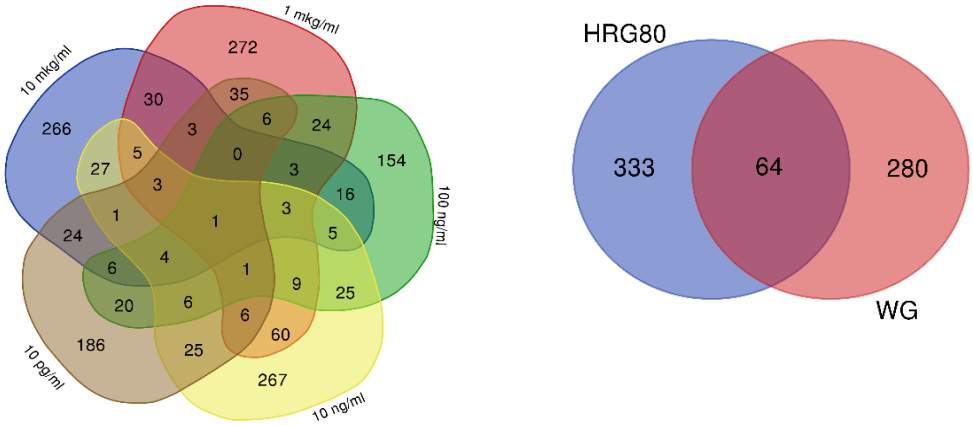
Table 1. The number of genes deregulated (>20 fold compared to control) by ginsenosides Rb1, Rg3, Rg5 and Rk1, WG and HRG80™ at different concentrations in the hippocampal neuronal cell line HT22.

Sample name	Concentration ng/ml	Molar Concentration **nM**	Content in WG **extract**/powder, %	Content in HRG80™ **extract**/powder %	Number of deregulated genes	Substance/ Concentration Specific genes
WG	10000	n/a	n/a	n/a	344	189
HRG1	10000	n/a	n/a	n/a	397	232
HRG2	1000	n/a	n/a	n/a	461	397
HRG3	100	n/a	n/a	n/a	283	246
HRG4	10	n/a	n/a	n/a	448	290
HRG5	0.01	n/a	n/a	n/a	327	169
Rb1	110.9	100	**4.250**/1.069	**0.183**/0.046	470	283*/279**
Rg3	78.5	100	**0.309**/0.080	**4.307**/1.080	413	235*/236**
Rg5	76.7	100	**0.031**/0.008	**7.534**/1.888	553	338*/345**
Rk1	76.7	100	**0.112**/0.028	**4.027**/1.009	373	215*/214**

* - compared to WG, ** - compared to HRG1



(**a**) (**b**)



(c) (d)

Figure 1. Venn-diagrams of: (a) - the number of genes deregulated by WG and ginsenosides Rb1, Rg3, Rg5 and Rk1, (b) - the number of genes deregulated by HRG and ginsenosides Rb1, Rg3, Rg5 and Rk1, (c) - the number of genes deregulated by HRG at the concentrations 10 μg/ml, 1 μg/ml, 100 ng/ml, 10 ng/ml, and 0.01 ng/ml; (d) - the number of genes deregulated by WG and HRG in the hippocampal neuronal cell line HT22.

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Only one gene, *SUOX*, encoding mitochondrial sulfite oxidase, was commonly deregulated by all purified ginsenosides, WG and HRG80 ™ (**Figure 1 a, b**) and gene KRTAP 10-7 in all tested concentrations of HRG80 ™ and total extract HRG80 ™ (**Figure 1c**.).

Deregulated genes expression profiles (signatures) of WG and HRG80 ™ are also significantly different; only 64 of 398 and 344 genes deregulated by HRG80 ™ (16% of total) and WG (18% of total) correspondingly were the same (**Figure 1d**).

These observations demonstrate that Red Ginseng preparation HRG80 ™ significantly impacts the gene expression of hippocampal neurons in a wide range of concentrations, from 1 μg/ml to 0.01 ng/ml.

These observations also reveal that the gene expression profile of hippocampal neurons is specific for every purified ginsenoside and quite different from Red Ginseng HRG80 ™ and WG preparations.

2.2. *Effect of ginsenoside Rg5 on signaling canonical pathways*

Figures 2 show the predicted effects (-log *p*-value > 1.3, z-score > 2) of Red and White Ginseng extracts (HRG80 ™ and WG) and their major constituents ginsenosides Rb1, Rg3, Rg5, and Rk1 on canonical signaling pathways, including inhibition of:

- neurotransmitters and nervous system signaling involved in ***neuroinflammation*** signaling (**Figures 3a),**
- ***senescence, ferroptosis, adrenomedullin,*** and ***WNT/β-catenin*** signaling are canonical pathways involved in cellular stress response, injury, and cancer progression (**Figures 3).**
- intracellular second messenger c-AMP mediated ***protein kinase A*** signaling (**Figure 3b),**
- transcription regulation ***sirtuin*** and nuclear receptors, ***estrogen receptors*** mediated signaling (**Figure 3b).**

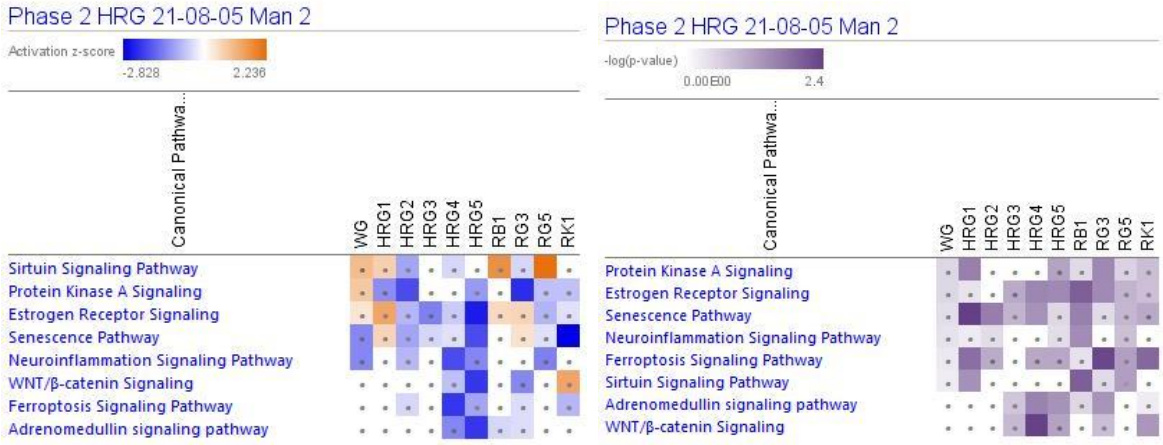

Figure 2. Effects of ginseng extracts WG (10 μg/ml), HRG at concentrations of 10 μg/ml (HRG1), 1 μg/ml (HRG2), 100 ng/ml (HRG3), 10 ng/ml (HRG4), and 10 pg/ml (HRG5), and ginsenosides Rb1, Rg3, Rg5, Rk1 at the concentration 100 nM on selected canonical pathways. The brown color shows the predicted activation, blue color – predicted inhibition of diseases; symbol · shows that the activation z-score was < 2 and the -log *p*-value <1.3 = $p < 0.05$. An absolute z-score ≥2 is considered significant activation (+) or inhibition (-). The activation z-score predicts the activation state of the canonical pathway, using the gene expression patterns of the genes within the pathway. The *p*-value calculated using a Right-Tailed Fisher's Exact Test indicates the statistical significance of the overlap of analyzed dataset genes that are within the pathway.

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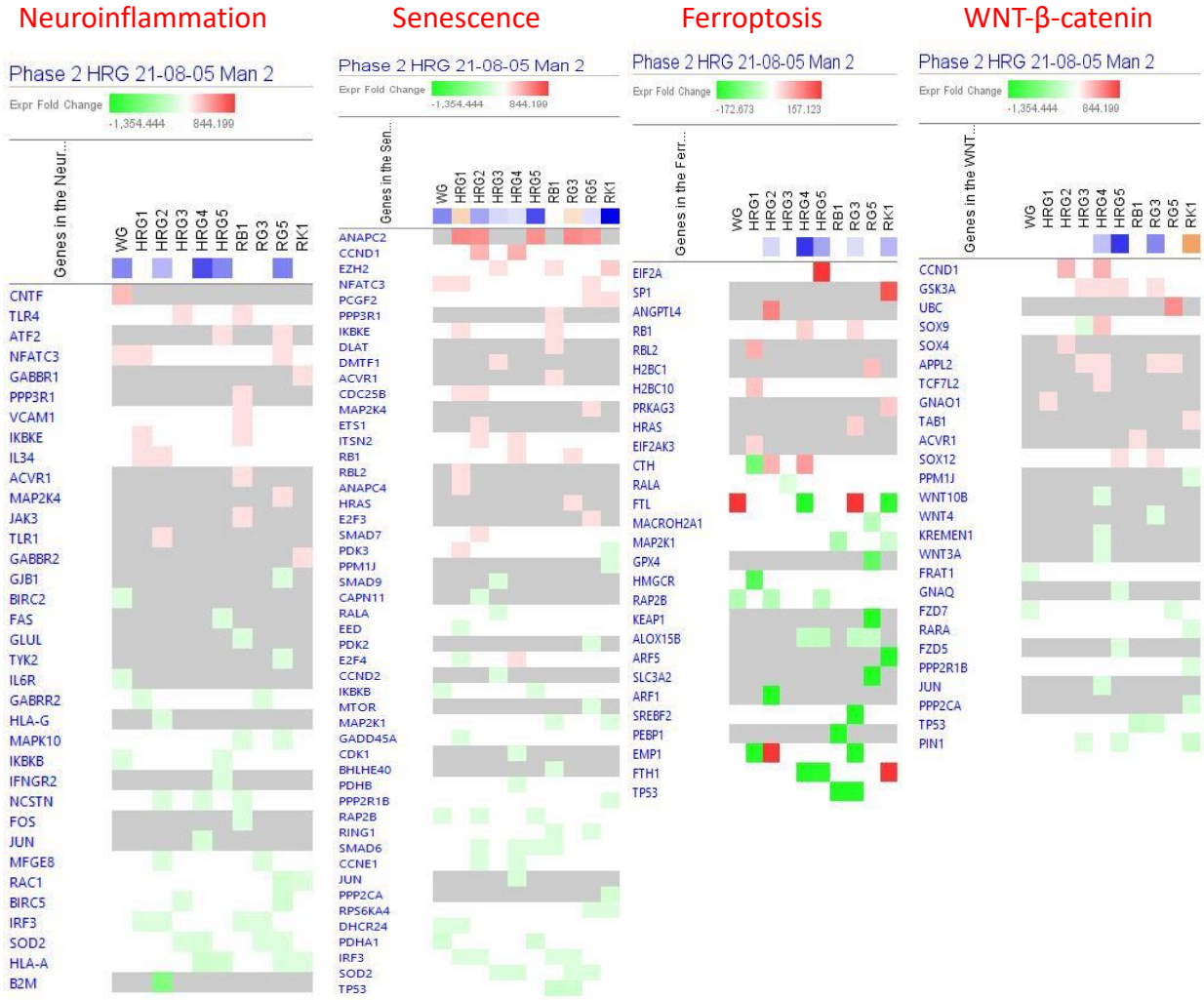

Figure 3a. Effects of WG at concentration 10 μg/ml, HRG at concentrations of 10 μg/ml (HRG1), 1 μg/ml (HRG2), 100 ng/ml (HRG3), 10 ng/ml (HRG4), and 10 pg/ml (HRG5), ginsenosides Rb1, Rg3, Rg5 and Rk1 at the concentration of 100 nM on neuroinflammation, senescence, ferroptosis, and WNT- β-catenin signaling pathways. The heatmap of gene expression (in fold changes compared to control, red—upregulation, green – downregulation), after exposure of cells with test samples at different concentrations; the column represents signatures of test samples with solid red or green squares indicating genes that are expected to be up-regulated down-regulated, respectively; color intensity indicates the actual log-fold changes.

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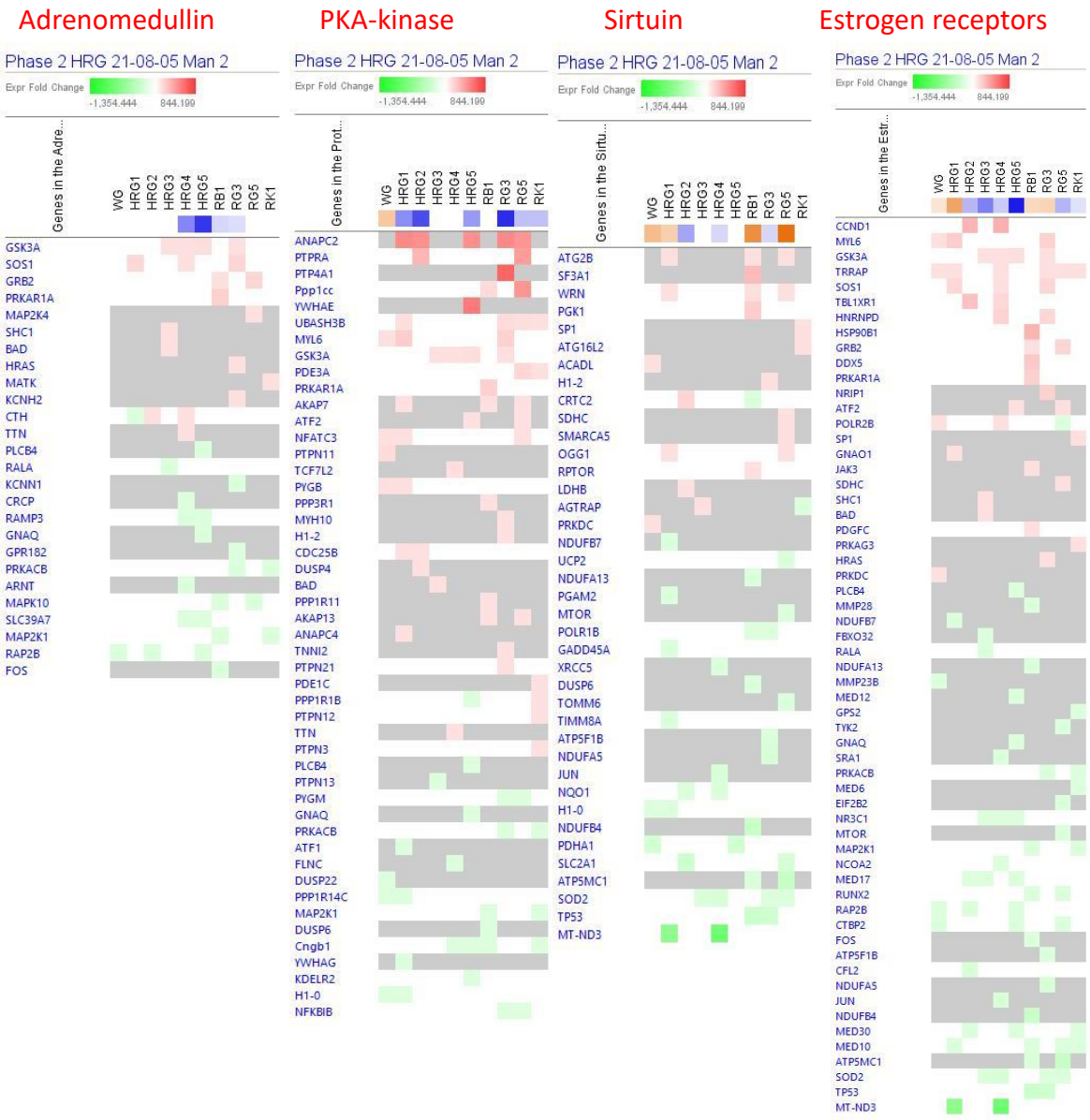

Figure 3b. Effects of WG at concentration 10 μg/ml, HRG at concentrations of 10 μg/ml (HRG1), 1 μg/ml (HRG2), 100 ng/ml (HRG3), 10 ng/ml (HRG4), and 10 pg/ml (HRG5), ginsenosides Rb1, Rg3, Rg5 and Rk1 at a concentration of 100 nM on adrenomedullin, PKA, sirtuin and estrogen receptors signaling pathways. The heatmap of gene expression (in fold changes compared to control, red—upregulation, green – downregulation), after exposure of cells with test samples at different concentrations; the column represents signatures of test samples with solid red or green squares indicating genes that are expected to be up-regulated down-regulated, respectively; color intensity indicates the actual log-fold changes.

The effects of ginseng extracts WG and HRG, and ginsenosides Rb1, Rg3, Rg5, and Rk1 on genes expression involved in these signaling pathways are shown in **Supplements 3-10** in detail.

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2.2. Predicted effects of ginsenosides and ginseng extracts HRG80 ™ and WG.

A large part (about 75%) of deregulated genes consist of networks that are significantly associated with cancer, gastrointestinal, and endocrine systems diseases and disorders, **Table 2** and **Figure 4**.

Table 2. The number of genes associated with various diseases and deregulated by ginseng extract HRG80 ™ and WG, and ginsenosides Rb1, Rg3, Rg5.

Test substance	**WG**	**HRG-80 ™**					**Rb1**	**Rg3**	**Rg5**	**Rk1**
Concentration, ng/ml	10000	10000	**1000**	100	10	0.01	100 nM	100 nM	100 nM	100 nM
Diseases and disorders										
Cancer	300	346	412	253	404	277	419	363	494	338
Gastrointestinal	259	308	360	222	357	243	370	317	439	301
Endocrine System	227	293	336	205	342	228	356	292	430	268
Neurological				167			265			
Reproductive System		237								
Hepatic									255	
Hematological					136					
Hereditary			**116**							
Infectious								77		
Developmental	68									
Metabolic						9				
Ophthalmic										9
Total genes	344	397	461	283	448	327	470	413	553	373

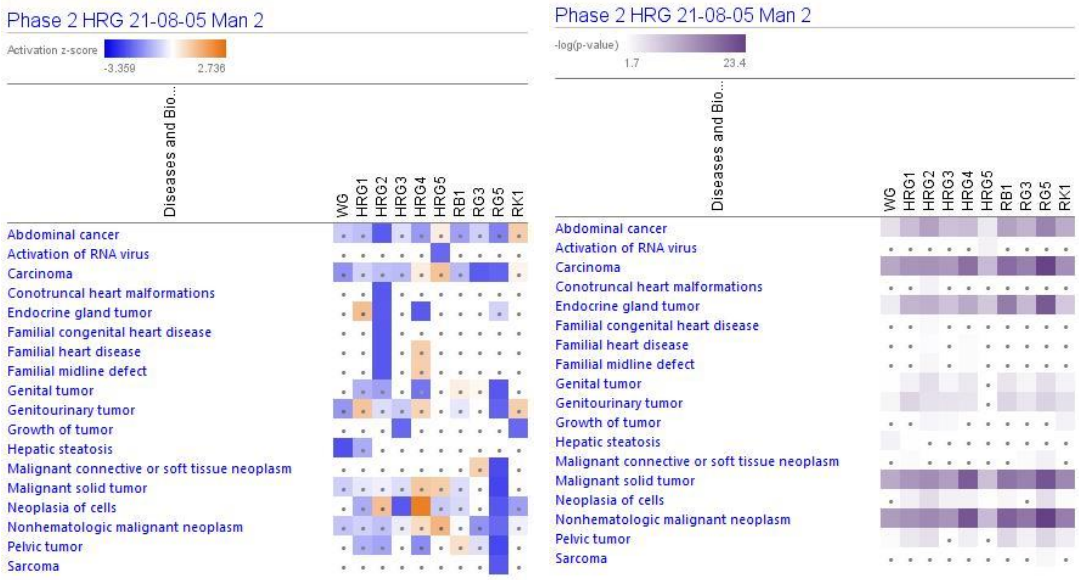

Figure 4. Effects of ginseng extracts WG (10 μg/ml), HRG80 ™ at concentrations of 10 μg/ml (HRG1), 1 μg/ml (HRG2), 100 ng/ml (HRG3), 10 ng/ml (HRG4), and 10 pg/ml (HRG5), and ginsenosides Rb1, Rg3, Rg5, Rk1 at the concentration 100 nM on diseases. Disease scores are presented using a gradient from dark blue to brown for predicted activation and light to dark blue for predicted inhibition of

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diseases; symbol · shows that the activation z-score was < 2 and the -log *p*-value <1.3 = $p < 0.05$. An absolute z-score ≥2 is considered significant activation (+) or inhibition (-).

IPA analysis shows diseases and bio functions (**Table 3**) that are expected significantly (activation score z > [±2], - log *p*-value) > 1.5) correlated by ginseng extracts HRG80™ and WG, and ginsenosides Rb1, Rg3, Rg5. HRG80™ is expected to inhibit endocrine gland tumors, abdominal cancer and neoplasm, necrosis, connective tissue cell death, and heart disease at the concentration of 1 μM.

At lower concentrations (100 or 10 ng/ml), HRG80™ may inhibit neoplasia of cells, invasion breast cancer cells lines, growth of tumor and endocrine glands tumor, while at the highest concentration (10 μg/ml) – to inhibit necrosis and proliferation of leukemia cell line.

Table 3. Predicted effects of ginseng extract HRG80™ and WG, and ginsenosides Rb1, Rg3, Rg5 in various diseases and bio functions expressed in the activation z-score values. Positive values show predicted activation, negative – predicted inhibition.

Test substance	**WG**	**HRG-80™**					**Rb1**	**Rg3**	**Rg5**	**Rk1**
Concentration, ng/ml	10000	10000	**1000**	100	10	0.01	100 nM	100 nM	100 nM	100 nM
Diseases and Biofunctions										
Abdominal cancer	-0.7	-0.867	**-2.162***	-0.447	-1.348	0.342	-1.274	-0.63	-1.664	0.922
Abdominal neoplasm	**-2.062***		**-2.018***							
Activation of RNA virus						**-2.0***				
Carcinoma	-1.437	-0.583	-0.849	-0.897	0.331	1.103	-0.92	**-2.16***	**-2.042***	0.206
Colorectal cancer cell viability										**-2.009***
Conotruncal heart malformations			**-2.2*** **-2.243***							
Connective tissues cells death										
Endocrine gland tumor		1.172	**-2.176***		**-2.186***				-0.594	
Familial congenital heart disease			**-2.2***							
Familial heart disease			**-2.2***		0.9					
Familial midline defect			**-2.2***		0.9					
Genital tumor		-1.039	-1.262		-1.838		0.3	0.135	**-2.207***	
Genitourinary tumor	-1.387	1.078	-0.444	-0.699	0.786		-0.319	-0.008	**-2.062***	0.875
Growth of tumor			0.048	**-2.012***						**-2.001**
Hepatic steatosis	**-2.313***	-1.102								
Invasion of breast cancer cell lines				**-2.014***						
Malignant neoplasm									**-2.002***	
Malignant solid tumor	-0.639	-0.322	-0.219	-0.452	0.99	0.904	-0.438	0.013	**-2.458***	0.039
Migration of tumor cells										**-3.101***
Necrosis		**-2.263***	**-2.594***				**-2.304***			
Neoplasia of cells		-1.108	1.18	**-2.195***	**2.348***	-0.7	-0.505		**-2.237***	-1.277
Nonhematologic malignant neoplasm	-0.799	-0.609	-0.821	-0.255	0.63	1.366	-0.087	-1.432	**-2.002***	-0.204
Pelvic tumor		-1.039	-1.191		-1.522		0.573	-0.417	**-2.415***	
Proliferation of leukemia cell line		**-2.070***								
Sarcoma									**-2.2***	

*- statistically significant effect, z-score > 2 and -log *p*-value > 1.3

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WG is expected to inhibit hepatic stenosis and abdominal neoplasm at the same concentration of 10 μg/ml. Antitumor activity profiles of purified ginsenosides are different from ginseng total extracts, where the most polyvalent is Rg5, which is expected to inhibit genital and genitourinary tumor, malignant neoplasm and solid tumor, neoplasia of cells, pelvic tumor, carcinoma, and sarcoma (**Table 3**).

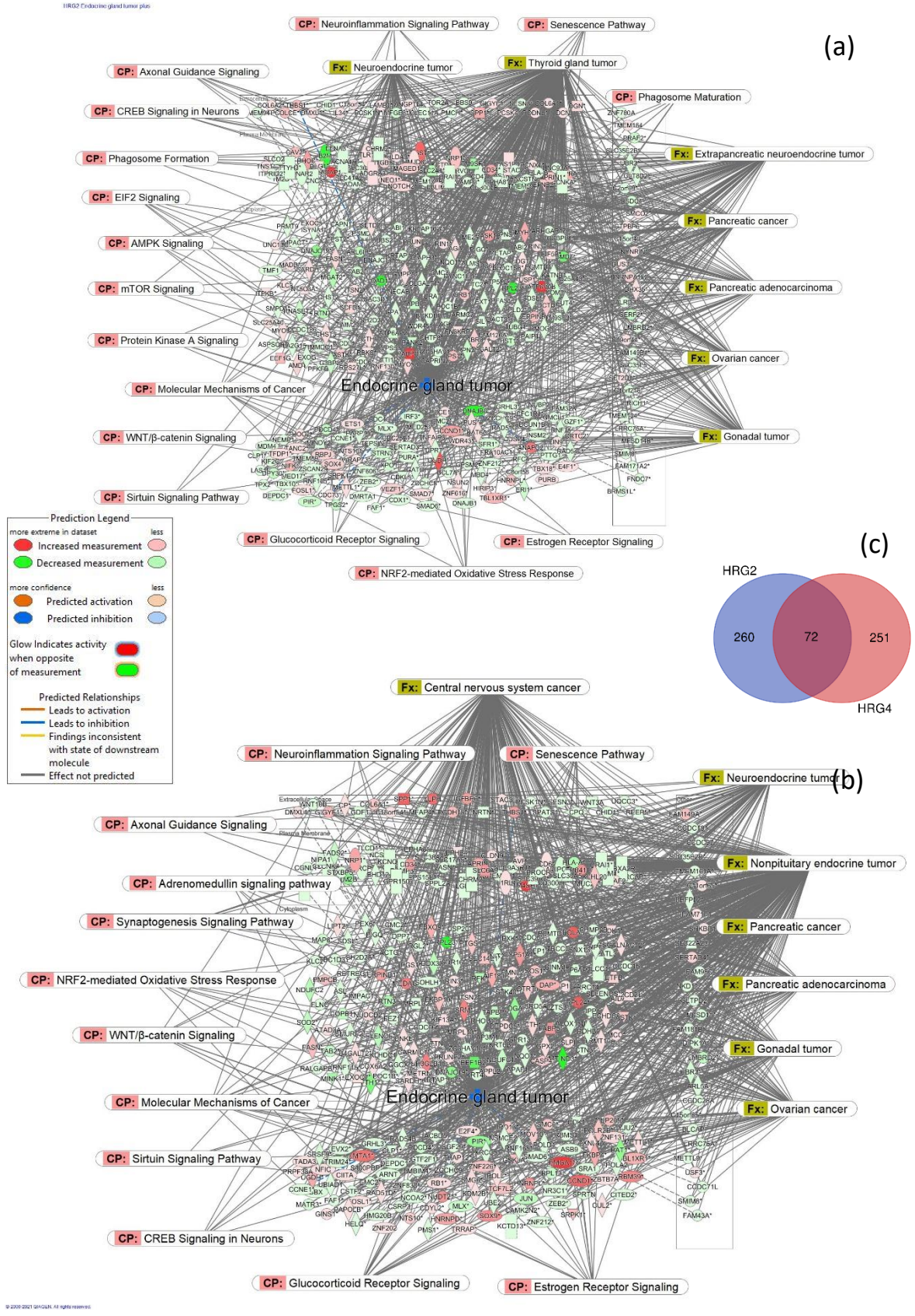

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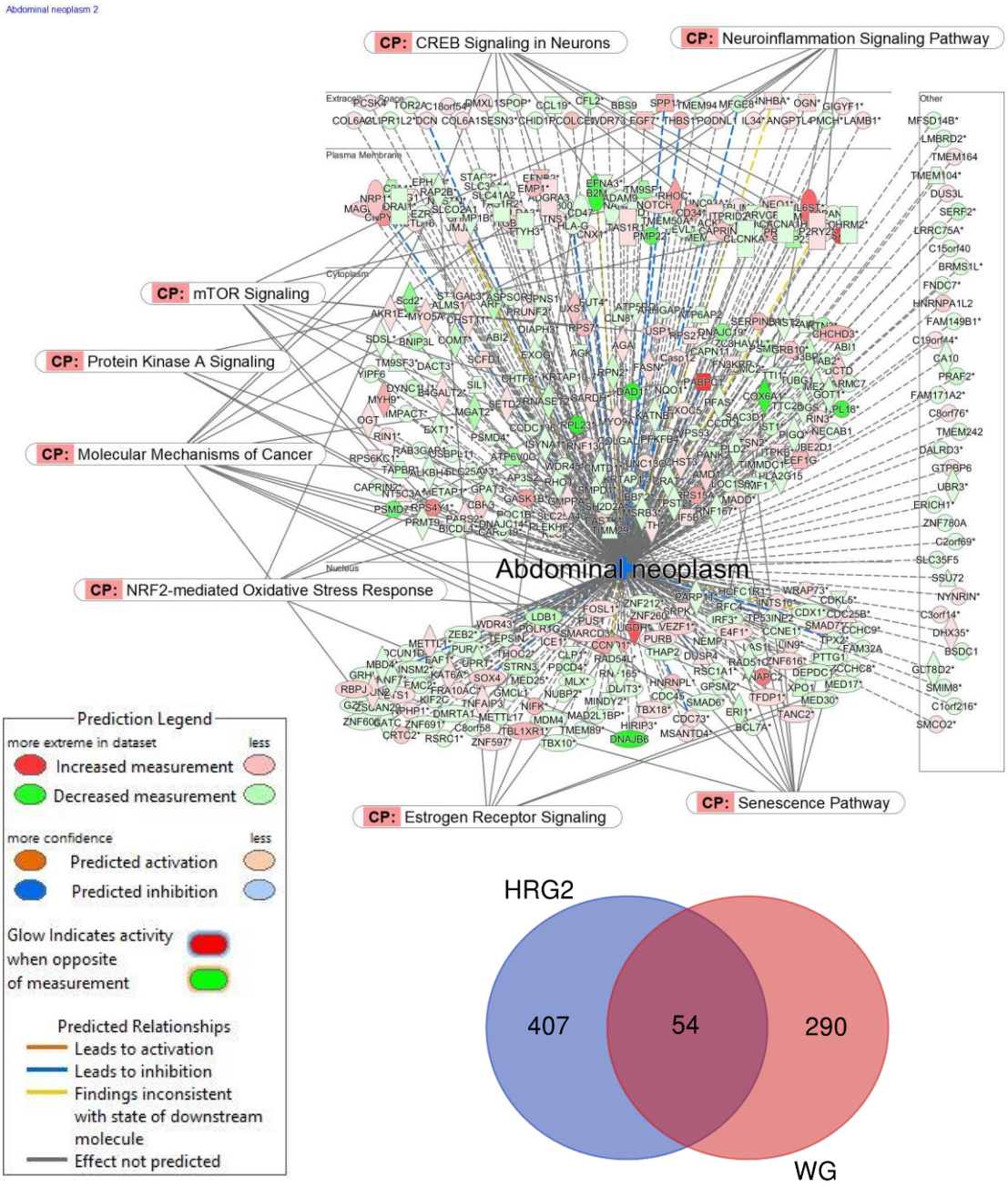
Figure 5. Molecular network shows predicted inhibition of exocrine gland tumor by HRG80 ^{TM} at concentrations: (a) - 1 μg/ml and (b) - 10 pg/ml. Solid red or green color nodes indicating genes that are up-regulated and down-regulated, respectively; color intensity indicates the actual log-fold changes. The tags labeled with purple display the canonical pathways related to particular genes of the network. The tags labeled with khaki show various types of tumors associated with the molecules in subnetworks. Figure (c) – Venn diagram showing the numbers concentration-specific and commonly deregulated (72) genes at concentrations 1 μg/ml and 10 pg/ml.

Expected Inhibition of endocrine gland tumors at two different concentrations 1 μg/ml and 10 pg/ml (**Figure 5**) is associated with two distinct networks (**Figure 5a** and **5b**), including quite different sets of molecules where 72 genes are commonly deregulated at these concentrations, while 250 and 251 are unique for each of them (**Figure** 5c and **Supplemental Table S3** in **Supplement 2**).

Similarly, expected Inhibition of abdominal neoplasm by red HRG2 and white WG ginseng is associated with two distinct networks (**Figure 6a** and **6b**), including quite different sets of molecules where 54 genes are commonly deregulated by HRG or WG, while 407 and 290 are unique for each of ginseng preparation (**Figure 6c**).



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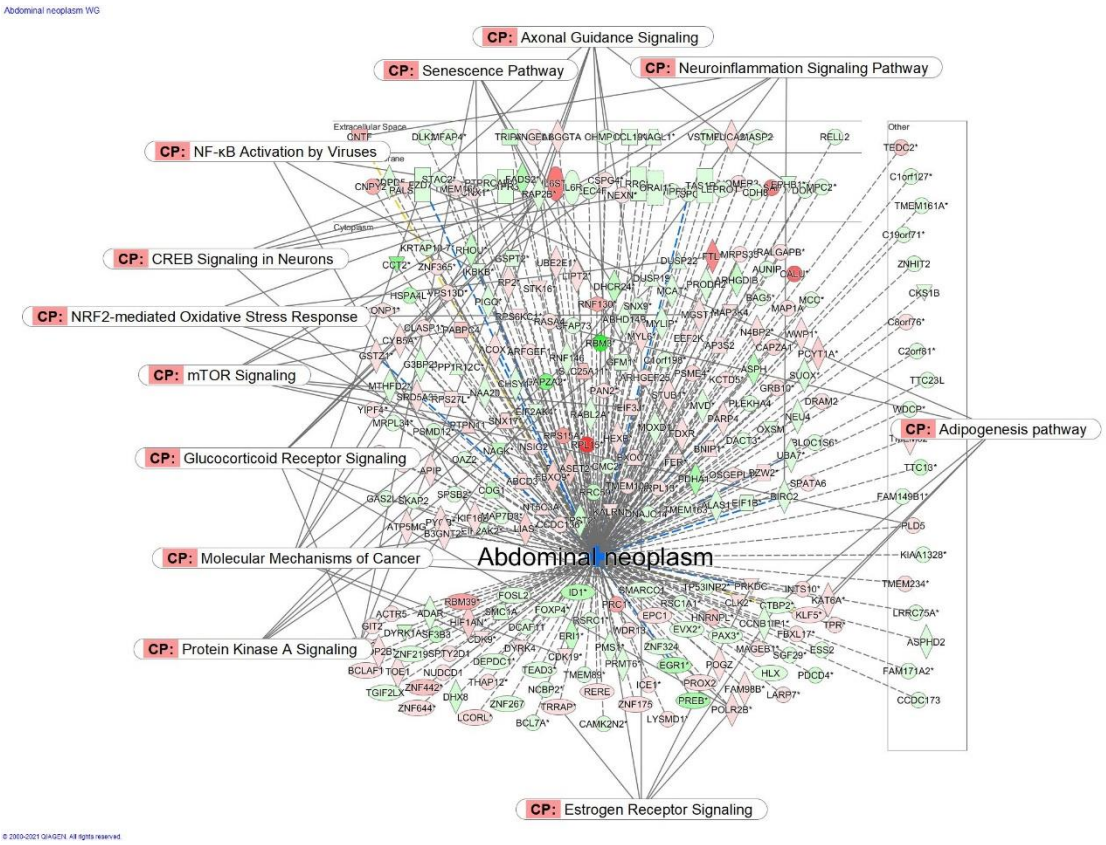

Figure 6. Molecular network shows predicted inhibition of abdominal neoplasm by: (a) - HRG80 ™ at concentration of 1 μg/ml and (b) – WG at concentration of 10 μg/ml. Solid red or green color nodes indicating genes that are up-regulated and down-regulated, respectively; color intensity indicates the actual log-fold changes. The tags labeled with purple display the canonical pathways related to particular genes of the network. The tags labeled with khaki show various types of tumors associated with the molecules in subnetworks. Figure (c) – Venn diagram showing the numbers product-specific and commonly deregulated 54 genes.

3. Discussion

This study's primary aim was to utilize transcriptomics of neuronal cells to uncover potential pharmacological activities and indications for use in medicine hydroponically cultivated Panax ginseng roots preparation HRG80™(HRG). This task is closely associated with exploring HRG at justified in vitro concentrations matching the concentrations of ginsenosides found in vivo studies on human subjects.

Our study provides evidence that HRG is pharmacologically active at the concentrations (10, 100, 1000, and 10000 ng/ml) corresponding to the concentrations of ginsenosides (from 0.05 to 35 nM inequivalent to 5 – 7000 ng/ml assuming that the content of ginsenoside Rg5 is 2% in the HRG80 ™, **Supplement 1**) detected in the blood of human subjects orally administered Red Ginseng preparations in the therapeutic dose [29].

At all tested concentrations, HRG deregulated genes significantly associated with cancer, gastrointestinal, and endocrine systems diseases and disorders, **Table 2 and Figure 4**.

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However, the expected (based on IPA analysis results) pharmacological profile and possible indications for use are different both for WG and HRG, as well as for various doses of HRG in blood. HRG80™ has the potential to inhibit endocrine gland tumors, abdominal cancer and neoplasm, necrosis, connective tissue cell death, and heart disease at the blood concentration of 1 μM, while at other concentrations, HRG80™ has the potential to inhibit neoplasia of cells, invasion breast cancer cells lines, growth of tumor and endocrine glands tumor, necrosis and proliferation of leukemia cell line. On the other hand, WG can presumably inhibit hepatic stenosis that is not expected for HRG.

These conclusions are in line with other reports on the antitumor activity of Ginseng and purified ginsenosides [19,23,25,26,36, 39-44].

Meanwhile, antitumor activity profiles of purified ginsenosides are different from Ginseng total extracts (**Table 3**). The results obtained for purified ginsenosides cannot be simply extrapolated on total extract or powdered roots, which exhibit quite different pharmacological and therapeutic activities.

Mechanism of action of HRG80™ is multitarget and associated with stress-induced abnormalities, neuroinflammation and senescence and the other signaling pathways (**Figures** 3-6) playing important role in these physiological and cellular processes, including apoptosis, tumorigenesis and progression of cancer, that is typical for adaptogens [8, 45,46].

Overall, these results of this study are in line both with traditional use of Ginseng in aging and the theory where low-grade chronic inflammation (inflammaging) that develops with senescence playing a crucial role in progression of aging related diseases, primarily cancer [47-49] and adaptogen concept suggesting efficacy of adaptogens in stress-induced aging related diseases [8, 50,51] (**Figure 7**).

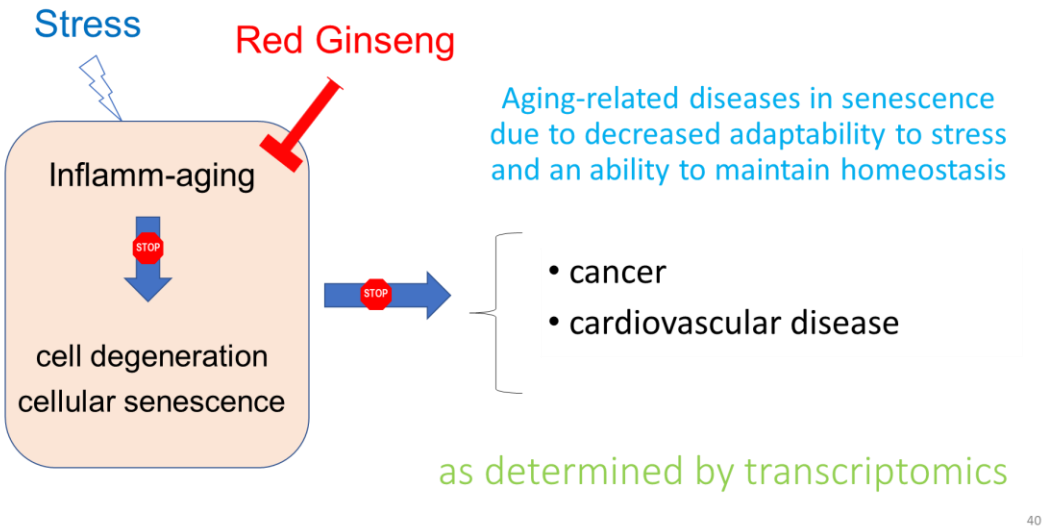

Figure 7. Hypothetical scheme of the effect of Ginseng on aging-related diseases as determined by transcriptomics

There are several constraints in this study. One of them is the lack of scientific literature about the direction (positive or negative) of correlations between gene expression and physiological function or disease for predicting effects of some experimental findings used *in silico* analysis. The second one is related to the number of concentrations points in the dose-response correlation study: more intermediate points in the range of 10 fold difference will show smooth changes from point to point.

Further preclinical and clinical studies are required for the assessment of the therapeutic efficacy of HRG80 ™ in different types of endocrine tumors, and abdominal cancers are needed.

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Overall, this is the first evidence of pharmacological activity of Ginseng preparations in the concentrations found in the blood of human subjects after oral administration in therapeutic doses.

4. Materials and methods

All materials and methods used in the present study have been described in detail in our previously published studies [52, 53]. Therefore, only a short description of herbal extracts, mRNA microarray hybridization, and Ingenuity Pathway Analysis (IPA) is provided below.

4.1. Test samples and reference standard

Powdered Red Ginseng preparation HRG80™ and its extract (HRG) were obtained at Botalys S. A. (Ath, Belgium). Harvested roots were air-dried and steamed to Red Ginseng. The Red Ginseng HRG80™ preparation was standardized for the content of the ginsenosides Rg1, Re, Rf, Rb1, Rg2, Rc, Rh1, Rb2, F1, Rd, Rg6, F2, Rh4, Rg3-(S-R), PPT (20-R), Rk1, C(k), Rg5, Rh2, Rh3, 20S-PPT, and PPD (**Supplement 11)**

Powdered Red Ginseng preparation HRG80™ and its extract (HRG) were obtained at Botalys S. A. (Ath, Belgium). Korean Ginseng (*P. ginseng* Meyer) root was hydroponically cultivated in controlled conditions, air-dried, steamed to Red Ginseng, which was powdered, and standardized for the content of ginsenosides Rg1, Re, Rf, Rb1, Rg2, Rc, Rh1, Rb2, F1, Rd, Rg6, F2, Rh4, Rg3-(S-R), PPT (20-R), Rk1, C(k), Rg5, Rh2, Rh3, 20S-PPT, and PPD to obtain Red Ginseng HRG80™ preparation containing 7.6% of total ginsenosides (**Supplemental Table S4** in **Supplement 11**). HRG80™ preparation was exhaustively extracted by 40% ethanol and evaporated to dryness to obtain HRG extract (DER 4: 1) used in vitro experiments. The content of ginsenosides in HRG extract was 30.32% (**Supplement 11**).

The reference standard, *P. ginseng* Meyer powdered root preparation, and the extract contained 5.57% and 22.15% total ginsenosides correspondingly (**Supplemental Table S4** in **Supplement 11**). All herbal preparations were analyzed and certified by Botalys S. A.

Purified reference standards of ginsenosides were purchased from Merck https://www.sigmaaldrich.com/. Working samples used in the experiments were prepared by diluting dimethylsulfoxide (DMSO) solution of the ginsenosides or Ginseng extracts with appropriate volumes of phosphate-buffered saline. The final concentration of DMSO in incubation media was the same as in the control – 1%.

4.2. mRNA microarray hybridization

Murine hippocampal neuronal HT22 cells were seeded and attached for 24 h prior to drug treatment. Cells were then treated for 24 h at various combinations and concentrations of drugs or DMSO as solvent control (0.5%). Total RNA was isolated using the InviTrap® Spin Universal RNA mini kit (250 preps; Stratec Molecular). RNA concentrations were determined using the NanoDrop spectrophotometer (NanoDrop Technologies). The quality of total RNA was confirmed by gel analysis using the total RNA Nanochip assay on an Agilent 2100 bioanalyzer (Agilent Technologies GmbH). Only samples with RNA index values > 8.5 were selected for expression profiling. The experiments were performed in duplicate for treated samples and for control samples by the Genomics and Proteomics Core Facility at the German Cancer Research Center in Heidelberg, Germany. For mRNA microarray hybridization the Affymetrix GeneChips® with mouse Clariom S assays have been used according to the manufacturer's instructions. Data analysis was done by normalization of the signals using the quantile normalization algorithm without background subtraction, and differentially regulated genes were defined by calculating the standard deviation differences of a given probe in a one-by-one comparison of samples or groups. The data were further processed using Chipster software (The Finnish IT Center for Science CSC).

4.3. Ingenuity Pathway Analysis (IPA)

Microarray data were analyzed by IPA (Ingenuity Systems). IPA software relies on the Ingenuity Knowledge Base, a frequently updated database containing biologic and chemical interactions and

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functional annotations gathered from the literature. In order to obtain information about cellular functions, networks, and affected pathways, IPA offers the Core Analysis tool, which was used for all datasets.

Using IPA, we performed different predictive algorithmic calculations on transcriptomic datasets, resulting in varying analyses, including (i) canonical pathways, which displayed the molecules of interest within well-established signaling or metabolic pathways; and (ii) upstream analyses, which predicted the upstream regulators (any molecule that can influence the transcription or expression of another molecule) that might be activated or inhibited to explain the expression changes in test datasets.

The interpretation of gene expression data was facilitated by consideration of prior biologic knowledge. IPA software relies on the Ingenuity Knowledge Base, a large gathering of observations with more than 8.1 million findings manually curated from the biomedical literature or integrated from 45 third-party databases. The network contains 40,000 nodes that represent mammalian genes, molecules, and biologic functions. Nodes are linked by 1,480,000 edges representing experimentally observed cause-effect relationships that relate to gene expression, transcription, activation, molecular metabolism, and binding. Network edges are also associated with a direction (either activating or inhibiting) of the causal effect [54].

To obtain information about the impact of test samples on cellular signaling pathways and networks for biologic functions and diseases downstream of the genes, whose expression has been altered in a dataset, we used the IPA Core Analysis tool for all tested datasets. Analysis of transcriptomics enabled us to predict regulators that are activated or inhibited based on the distinct up- and downregulation patterns of the expressed genes, and to determine which causal relationships previously reported in the literature are likely to be relevant for the biologic mechanisms underlying the data.

4.4. Statistical analysis

Two statistical methods of analysis of gene expression data were used in Ingenuity Pathway analysis: (a) Gene-set-enrichment method, where differentially expressed genes are intersected with sets of genes that are associated with a particular biological function or pathway providing an 'enrichment' score [Fisher's exact test p-value] that measures overlap of observed and predicted regulated gene sets [55,56]; (b) The method that based on previously observed cause-effect relationships related to the direction of effects reported in the literature [57, 58] providing so-called z-scores assessing the match of observed and predicted up/down-regulation patterns [55.56] . The predicted (z-score > 2; or -log *p*-value >1.3) effects are based on changes of gene expression in the experimental samples relative to the control.

5. Conclusions

In this study, we, for the first time, have demonstrated that the gene expression profile of murine hippocampal neuronal HT22 cells is changed significantly in response to exposure of hydroponically cultivated Red Ginseng preparation HRG80 ™ in concentrations ranging from 10 μg/ml to 0.01 ng/ml. That is in line with concentrations of ginsenosides Rb1, Rg3, Rg5, and Rk1 found in vivo studies on human subjects taking orally therapeutic doses of Red Ginseng. Purified ginsenosides Rb1, Rg3, Rg5, and Rk1 have substantially various effects on gene expression profiles (signatures) and predicted pharmacological activities as determined by *in silico* analysis of transcriptomics. Their signatures are different from the signatures and expected therapeutic indications of Red Ginseng HRG80 ™ and white ginseng preparations, which are also quite different.

Comparative *in silico* transcriptome analysis of microarray-based gene expression profiles of neuronal cells exposed to Red and White Ginseng extracts and their major constituents ginsenosides Rb1, Rg3, Rg5, and Rk1 predicts a potential beneficial effect in neuroinflammation, senescence, and cancer, gastrointestinal, and endocrine systems diseases, and disorders. HRG80 ™ has the potential to inhibit endocrine gland tumors, abdominal cancer and neoplasm, necrosis, connective tissue cell death, and heart disease at the blood concentration of 1 μg/ml.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Supplementary data 1-11

Author Contributions: SA contributed to the execution of experiments, data management, and manuscript preparation. TE contributed to the overall management of the study and manuscript preparation. AP contributed to the study conception, design and evaluation, data analysis, interpretation of the results, and drafting and preparation of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: AP has an independent-contractor agreement with EuroPharmaUSA Inc. All other authors declare no competing interests. The funders had no role in the study's design, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

Ethical Statement: No ethics committee's approval is required in Germany for *in vitro* experiments of commercially available murine hippocampal neuronal HT22 cells. The author is responsible for all facets of the manuscript in ensuring that issues related to the accuracy or integrity of any part of the study are appropriately addressed and settled.

Appendix A. Supplementary data 1-11.

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