GHK, the Human Skin Remodeling Peptide, Induces Anti-Cancer Expression of Numerous Caspase, Growth Regulatory, and DNA Repair Genes

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Abstract: GHK (glycyl-L-histidyl-L-lysine) is a human plasma copper-binding peptide that declines during aging. Numerous studies have established many biological actions of GHK: it improves tissue regeneration, possesses anti-oxidant and anti-inflammatory effects, increases cellular stemness; increases decorin, angiogenesis, and nerve outgrowth. In recent studies, GHK was found to switch gene expression from a diseased state to a healthier state for certain cancers and for chronic obstructive pulmonary disease. In studies of aggressive, metastatic human colon cancer, the Broad Institute's Connectivity Map indicated that GHK, out of 1,309 bioactive molecules studied, reversed the expression of 70% of 54 genes over-expressed genes. GHK also reactivates programmed cell death in several cultured human cancer lines.

To determine GHK's potential as a cancer treatment, we analyzed the molecule's effect on the human gene expression using the Connectivity Map. GHK induces a 50% or greater change of expression in 31.2% of human genes. GHK increased gene expression in 6 of the 12 human caspase genes that activate programmed cell death. In 28 other genes, GHK altered the pattern of gene expression in a manner that would be expected to inhibit cancer growth. For DNA repair genes, there was a one-sided increase in the expression of such genes (47 UP, 5 DOWN).

A previous study found that a copper peptide plus ascorbic acid inhibited Ehrlich ascites cancer in mice. Using this method with GHK-copper gave a strong suppression of Sarcoma 180 in mice. These results support the idea that GHK may help to impede or suppress cancer growth.

Keywords: Copper peptides, cancer therapy, cancer inhibition, sarcoma, connectivity map.

INTRODUCTION

Despite advances in cancer diagnosis and treatment, many aspects of cancer growth and progression remain a mystery. As a result**,** current therapeutic approaches such as chemotherapy, surgery and radiation therapy are not only costly with severe side effects, but often produce disappointing results. For example, for colorectal cancer, one of the most common cancers worldwide, about 20% of patients develop metastatic disease and in 30% of cases cancer reoccurs after surgery [1]. It is generally established that cancer growth is associated with genetic alterations leading to activation of oncogenes and inactivation of tumor suppressors. Today, gene expression profiling studies are increasingly used to gain better understanding of cancer biology and to identify candidate therapeutic compounds [2]. However, computer gene profiling without connection to biological and clinical data has limited value. It is not enough for a candidate compound to effect gene expression in cell culture, it should also have well-documented positive biological effects in animal and clinical studies as well as a demonstrated history of safe usage.

The human tripeptide GHK (glycyl-L-histidyl-L-lysine) has been extensively studied since its discovery in 1973. GHK was originally isolated during studies on human aging as a low-molecular weight blood plasma activity that supported cells and tissues but which declined with age. GHK was isolated as the causative factor and was higher in the plasma of 20-25 year-old healthy men (200 micrograms/L) than in healthy men of 50-70 (80 micrograms/L) [3]. GHK has many biological actions: healing of dermal wounds; induction of systemic wound healing; remodeling of skin, enlargement of hair follicles; healing of gastric ulcers, duodenal ulcers, and bony tissue; anti-oxidant and anti-inflammatory activities**;** increased production of decorin, angiogenesis, and nerve outgrowth as well as the stemness of human keratinocytes [4–9]. Furthermore, in fibroblasts from patients with severe emphysema, GHK shut down tissue destructive genes while increasing the output of genes associated with tissue healing and remodeling [10].

In 2010, Hong *et al.* identified a metastatic-prone signature for aggressive early stage mismatch-repair colorectal cancer consisting of a 54 gene-set yielded by

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the best classification model [11]. The Broad Institute's Connectivity Map, a compendium of transcriptional responses to compounds, which contains data on 1,309 bioactive molecules, was used to find compounds that reverse the differential expressions of these genes, suggesting they may have a therapeutic effect on the metastasis-prone patients [12]. The results indicated that two wound healing and skin remodeling bioactive molecules, GHK at 1 micromolar and securinine at 18 micromolar, could significantly reverse the differential expression of these genes. Functioning at such low dosages makes these two small molecules ideal candidates for adjuvant chemotherapeutics.

Normal healthy cells have checkpoint systems to self-destruct if they are synthesizing DNA incorrectly through the programmed cell death or apoptosis system. Subsequently, Matalka *et al.* demonstrated that GHK, at 1 to 10 nanomolar, re-activated the apoptosis system, as measured by the caspases 3 and 7, and inhibited the growth of human SH-SY5Y neuroblastoma cells, human U937 histiocytic lymphoma cells, and human breast cancer cells as confirmed by Tino Unlap (personal communication, October 2013). In contrast, the GHK accelerated the growth of healthy human NIH-3T3 fibroblasts [13].

Cancer is primarily a disease of the elderly and GHK was discovered during a search for the reason blood from younger mammals, including humans, is more tissue supportive than blood from older mammals. To unify and clarify these observations on cancer, and determine the possible scope of GHK's potential as a cancer treatment, we analyzed the molecule's effect on the human genome using the Broad Institute's GenePattern and Connectivity Map. In addition, we investigated the anti-tumor potential of GHK using mice implanted with Sarcoma 180 cancer cells.

METHODS

Gene-Expression Analysis

Our raw data was acquired from the Broad Institute's Connectivity Map which is a repository of microarray experiments consisting of 7000 genome-wide expression profiles of 4 human cell lines treated with 1,309 bioactive small molecules for a total of 6,100 instances. This tool allows us to connect diseases with genes and drugs that may treat them through an in silico method. The Connectivity Map contains three gene expression profiles for GHK that were created using the GeneChip HT Human Genome U133A Array (data was retrieved March 5, 2013). Two of the profiles were created using the PC3 cell line while the third used the MCF7 cell line. All three profiles were combined. GenePattern was utilized to process CEL files with MAS5 and background correction. Files were then uploaded to the Comparative Marker Selection Viewer module in order to view fold changes for each probe set.

Sifting through all the probes/genes and combining duplicates (probe sets mapping to the same gene), we determined that there are 13,424 genes in the Broad data represented by 22,277 probe sets. This implies that each gene, on average, is measured by 1.66 probe sets and we use this number to estimate the number of genes affected by GHK at various cutoff points (rather than probe sets). The Broad's GenePattern produces gene expression as fold-changes in m-RNA production. The fold changes of genes with multiple corresponding probes were converted to percentages and then averaged.

Mouse Tumor Studies

The only animal study on the use of a copper tripeptide to inhibit cancer growth was from Linus Pauling's research group. This study used a combination of the Gly-Gly-His: copper 2+ and ascorbic acid to inhibit the growth of an Ehrlich ascites cancer in mice [14].

For our study, we followed the methods and dosages of the above Ehrlich ascites experiment but used a solid tumor (mouse Sarcoma 180) made up from a passage mouse to furnish 2 x 10^6 viable cells per 0.1 ml inoculum. The cells were injected into the right hamstring muscle mass of male Swiss Webster mice by methods previously published [15]. The tumor was allowed to establish for 3 days before treatment started. No mice died or were removed from the experiment.

Two therapeutic solutions were tested after adjusting pH to 7.0 by adding concentrated sodium carbonate solution. Number 1 solution was an aqueous mixture of 10^{-3} M GHK- copper 2+ and 4% ascorbate. Number 2 solution was an aqueous mixture of 10^{-3} M GHK-copper 2+, 10^{-3} M L-Histidine, and 4% ascorbate.

After the establishment of the tumor, one group of 13 mice were injected intraperitoneally with 0.2 ml of the Number 1 solution. Similarly, 0.2 ml of the Number

2 solution was injected into seven mice and also at 0.5 ml into another seven mice. Control (10 mice) and Tumor-only mice (8 mice) were injected with physiological saline. The mice were injected each Monday, Wednesday, and Friday over the next 14 days; the experiment was subsequently terminated by day 20. The mice were terminally anesthetized and their right rear legs were amputated at the thigh. Skin was removed to expose the site of the tumor. The net tumor weight was determined by subtracting the mean value of the legs of the control mice. The Student T-test statistic was used to determine P values.

RESULTS

Table **1** is an estimate of the number of genes affected by GHK at the various cutoff points. This is the probe set number divided by 1.66 since many probes bond to the same gene. The number of genes affected by GHK with a percent change greater than or equal to 50% UP or DOWN is 4194/13,424 or 31.2%. See Table **1**.

Table 1: Estimate of Number of Genes Affected by GHK

Percent Change	Genes Stimulated	Genes Suppressed
50-99%	1569	583
100-199%	646	469
200-299	227	196
300-599%	196	207
600-899%	39	42
900-1199%	8	7
1200% or more	2	4

GHK, Genes, and Cancer Suppression

Caspases are key activators of apoptosis. The data obtained by Matalka *et al.* measured GHK's effect on caspases 3 and 7 [13]. GenePattern indicates that GHK increases gene expression in 6 of the 12 caspase human proteins plus the NLPR1 gene that encodes Ced-4 apoptosis proteins and the BCL2L14 apoptosis facilitator.

GHK increases m-RNA, presumably the proteins, of putative cancer suppressors P63, P73, CTNNA1, APC, PAWR, ING2, IL15, IL25, BCL2L14, and AANAT. BRCA1 is mildly increased. Alternatively, GHK suppresses possible cancer-supportive genes FGFR2, TNF, and IGF1. The gene that produces decorin was increased only 44% but GHK-copper treatment of rat wound chambers increased the m-RNA for decorin 302%, suggesting GHK actions are modified by other regulators [16] see Tables **2-4**.

GHK and DNA Repair Genes

Using NetAffx Analysis Center (http://www.affymetrix.com), the annotations for each probe set in the GeneChip HT Human Genome U133A Array were retrieved. Searching the Gene Ontology (GO) descriptions for the term *DNA Repair* resulted in 497 probe sets (313 genes).

GHK stimulates many DNA repair genes. In the studied range (50% or more changes), gene expression is increased in 47 genes and decreased in 5 genes see Tables **5** and **6**.

Cell culture results also suggest that GHK activates DNA repair. Anti-cancer radiation therapy damages

Table 2: GHK and Gene Expression in Apoptosis Proteins

Genes	Percent Change in Gene Expression	Comment
CASP 1	432	Caspase proteins activate programmed cell death [13]
CASP 3*	65	
CASP 6*	23	
CASP 7*	48	
CASP 8	399	
CASP 10	195	
NLRP1	249	Apoptosis caspase recruitment domain [17]
CARD10	173	Apoptosis signaling gene [18]
BCL2L14	153	Apoptosis facilitator [19]

*Expanded range to include all probes with changes greater than 35% in order for comparison among all caspases. All others are probes with changes greater than 100%.

Table 3: GHK and Gene Expression in Cancer Suppressors

Genes	Percent Change in Gene Expression	Comment
USP29	1056	Ubiquitin specific peptidase 29, May stabilize P53 tumor suppressor [20]
IFNA21	955	Combined treatment of IFN-alpha and IL-21 increases anti-cancer effects [21]
TP73	938	Tumor suppressor [22]
TP63	Uncertain	Tumor suppressor. Gene probes are inconsistent however, TP63 was induced by GHK in keratinocyte cells in skin equivalent organ culture [8]
LEFTY2	935	Inhibition of pancreatic cancer cells [23]
IL25	891	Inhibits breast cancer cell growth [24]
IL15	875	Induces natural killer cells Anti-tumor and anti-viral [25]
D4S234E	731	p53-responsive gene, induces apoptosis in response to DNA damage [26]
MTUS2	474	Microtubule associated tumor suppressor [27]
C13orf18	352	Inhibits cervical cancer cells [28]
ING2	337	Functions in DNA repair and apoptosis [29]
CTNNA1	336	Suppresses cancer invasion of tissues [30]
CDKN1C	277	Breast cancer inhibitor [31]
PAWR	199	Induces apoptosis in cancer cells [32]
APC	195	Suppresses colon cancer [33]
PTEN	165	Cancer suppressor [34,35]
NRG1	164	Cancer suppressor [36]
NF1	143	Neurofibromin 1 [37]
ATM	107	Senses DNA damage [38]
ING4	107	Cancer suppressor
DCN*	44	Suppresses cancer growth and metastasis [39]. In rat wound chamber experiments GHK increased mRNA for decorin 302% [16]
BRCA1*	44	Cancer suppressor [40]

*DCN is included in this table due to its large increase observed in rat wound chamber experiments. BRCA1 is included in this table due to immense interest in the gene.

Table 4: GHK and Gene Expression in Cancer Enhancers

Genes	Percent Change in Gene Expression	Comment
ABCB1	-1537	Increases drug resistance in cancer cells [41]
STAT5	-982	Signals cancer cells to grow [42]
FGFR2	-904	FGFR2 inhibitors reduce some cancers [43]
FAIM2	-749	Prevents apoptosis [44]
IGF1	-522	Risk factor for cancer [45]
TNF	-115	May promote cell cancer invasion [46]

Table 5: GHK is Primarily Stimulatory on DNA Repair Genes

Percent Change in Gene Expression	Genes UP	Genes DOWN
50% - 100%	41	4
100% - 150%	2	1
150% - 200%	1	0
200% - 250%	2	0
250% - 300%	1	0

Table 6: Most Affected DNA Repair Genes

UP	Gene Title	Percent Change in Gene Expression
1	poly (ADP-ribose) polymerase family, member 3, PARP3	253
2	polymerase (DNA directed), mu, POLM	225
3	MRE11 meiotic recombination 11 homolog A MRE11A	212
4	RAD50 homolog (S. cerevisiae), RAD50	175
5	eyes absent homolog 3 (Drosophila), EYA3	128
6	retinoic acid receptor, alpha, RARA	123
DOWN		
1	cholinergic receptor, nicotinic, alpha 4, CHRNA4	-105

cellular DNA and slows cell replication. GHK-Copper at 1 nanomolar restored normal growth rates to radiation damaged cells [47].

Tumor Inhibition Studies

The mixture of GHK-Copper 2+ and ascorbic acid markedly inhibited (-60%) tumor growth. Since copper 2+ has six binding electronic orbitals and GHK only binds to three orbitals, we added L-histidine to add another bound molecule. In theory, this should stabilize the complex. Surprisingly, this potentiated the anti-tumor activity and no tumors were evident, at least by weight measurements see Table **7** and Figure **1**.

DISCUSSION

There is extensive published biological data on GHK's effects in both human and animal studies on tissue regeneration and protection. We consider the biological data in cell culture and animals as the most dependable.

The gene response demonstrated by m-RNA data is of a more suggestive nature since m-RNA produces proteins and peptides but has many secondary controls. The m-RNA may be translated into proteins 1 to 500 times and the subsequent proteins and peptides often undergo further modification. And then there is the influence of about 4 million regulatory micro-RNA molecules, whose action is still poorly understood. Furthermore, different cell types may produce somewhat different patterns of gene response to GHK. Nonetheless, the Broad data predictions that GHK would reverse the gene disease profiles of colon cancer and COPD were confirmed in affected cells where the gene disease pattern was moved to a healthier state [10,11].

In the Broad system, GHK increases expression in 2,686 genes by 50% or more and decreases it in 1,508 genes. Iorio *et al.* used the Connectivity Map, and MANTRA software (www.mantra.tigem.it) to explore networks of compounds producing similar transcriptional responses. GHK altered gene expression in a set of genes called Community 15 and increased transcription in 268 of those genes while suppressing 167 [48]. This may represent genes more exclusively controlled by GHK.

During wound healing studies in rats, mice and pigs, copper-free GHK produced a very mild stimulation of healing, but the addition of copper 2+ to GHK strongly enhanced repair. The interaction of GHK with copper

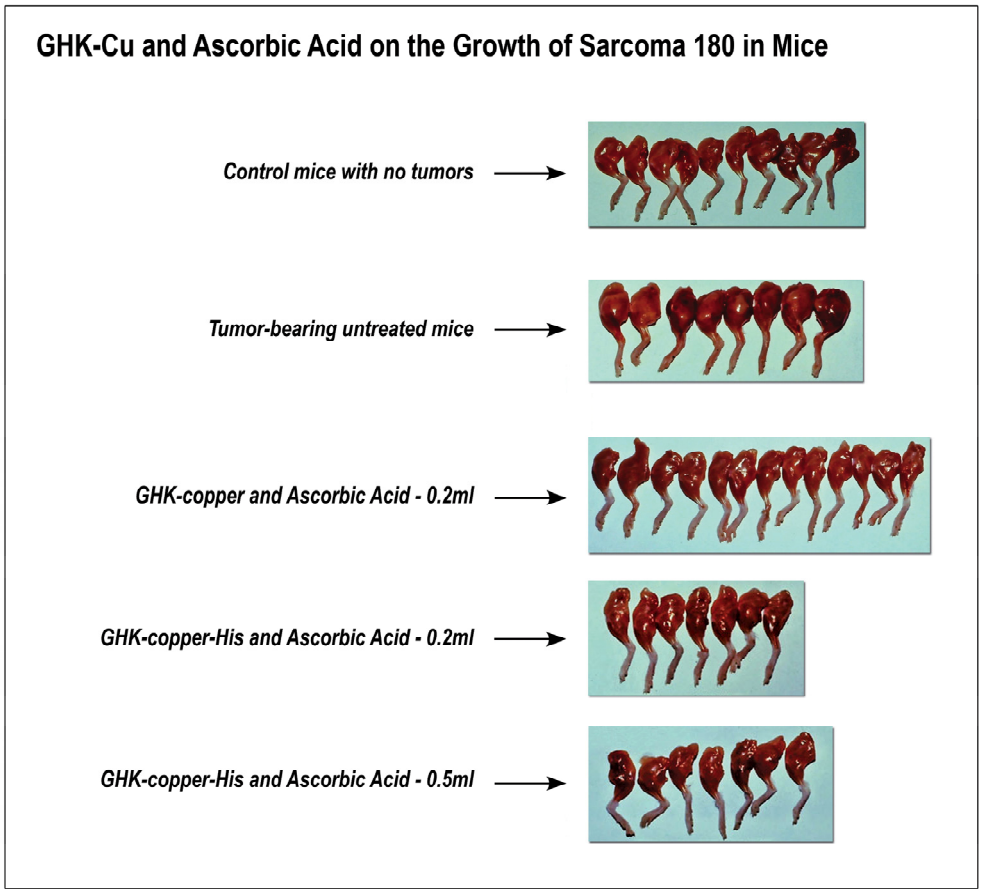

Figure 1:

Table 7: Inhibition of Mouse Sarcoma 180 - Weights and P Values

Group	Control Group No Tumor	Tumor-only No Treatment	GHK-Copper plus Ascorbic Acid 0.2 ml injected	GHK-Copper-His plus Ascorbic Acid 0.2 ml injected	GHK-Copper-His plus Ascorbic Acid 0.5 ml injected
Number Mice	10	8	13	7	7
Average Tumor Weight	0	3.64 grams	1.62 grams	0.06 grams	-0.17 grams
P value vs Control	x	P<0.000002	P<0.001	Not Significant	Not Significant
P value vs Tumor Only	P<0.000002	x	P<0.0002	P<0.0001	P<0.0001

2+ is important and it is likely that a mixture of GHK and GHK-copper 2+ exists in cell culture and *in vivo* experiments. GHK has an extraordinary affinity for copper 2+ (pK of association is 16.4), and can obtain copper 2+ from albumin (pK of association is 16.2). Equilibrium dialysis studies demonstrated that GHK easily exchanges copper 2+ with albumin. Furthermore, ternary complexes, such as GHK-copper-histidine can be formed raising the stability of the copper complex. However, in human plasma there is a 700-fold molar excess of albumin over GHK, and the tripeptide makes only a minor contribution to overall copper 2+ transport [4].

The results suggest that GHK acts in a manner which may help to slow cancer growth. We found that GHK affected 84 genes (general and DNA repair) that may help control abnormal cancerous growths. While

we recognize that cancer growth controls are often uncertain, these results suggest a variety of genes may serve to inhibit cancerous cell replication, at least early in life. During wound healing, GHK has tissue remodeling actions, the phase of healing in which cell migration into the wound area is stopped and cellular debris removed. The anti-tumor actions of the molecule may be related to these types of GHK effects.

The gene results plus culture experiments indicate that GHK reactivates the programmed cell death system in cancer cells and may activate other systems. Matalka *et al.* found GHK, at 1 to 10 nanomolar, re-activated the apoptosis system, as measured by the caspases 3 and 7. However, gene expression results indicate that GHK activates 6 of the 12 human caspase genes.

DNA repair mechanism activity declines with age and this contributes to cancer development and senescence [49-51]. At the 50% UP or DOWN cutoff point, there are 47 DNA repair genes increased and 5 decreased.

The only published prior evidence on copper peptides inducing anti-cancer actions in mammals is with the use of a similar copper peptide, Gly-Gly-His:copper 2+, combined with vitamin C to inhibit the growth of an Ehrlich Ascites cancer in mice [14]. Our studies in mice found the mixture of GHK-Copper 2+ and ascorbic acid to strongly suppress the growth of mouse Sarcoma 180.

While cancers are extremely diverse, it bears noting that GHK is highest in healthy young men of ages 20-25, a group with very low cancer rates. There are a variety of late-onset, inherited, genetic diseases that arise after age 40 to 60. This raises the question, "What is preventing these diseases from arising earlier?" For example, BRCA1 is a protective anti-cancer gene unless it is mutated. Yet, the initiation of the cancer is often delayed for 40 to 60 years. It is likely that multiple other genes inhibit the cancer development, but during aging these genes are silenced as are their protective actions.

GHK as a Clinical Treatment

GHK, abundantly available at low cost in bulk quantities, is a potential treatment for a variety of disease conditions associated with human aging. The molecule is very safe and no issues have ever arisen during its use as a skin cosmetic.

Based on our mouse tumor studies in which GHK-copper 2+ or GHK-copper 2+-Histidine was injected intraperitoneally, to inhibit tumor growth, we estimate about 100-200 mgs of GHK-copper 2+ or GHK-copper 2+-Histidine may inhibit tumor growth in humans. But even this may overestimate the effective dosage of the molecule. Most cultured cells respond maximally to GHK at 1 nanomolar. GHK has a half-life of about 1 hour in plasma and two subsequent tissue repair studies in rats found that injecting GHK intraperitoneally 10-times daily lowered the necessary dosage by approximately 100-fold in contrast to our earlier previous studies. While these tumor studies are not a direct comparison to GHK's effects, the result does suggest that GHK-Copper 2+ could be of value in some types of cancer therapy [52,53].

The use of portable continuous infusion pumps for a treatment might maintain this level in the plasma and extracellular fluid with much less GHK. Possibly the peptide could be administered with a transdermal patch [54]. Another approach could be to use peptide-loaded liposomes as an oral delivery system for uptake into the intestinal wall without significant breakdown [55,56].

Most current drugs being developed as cancer therapies tend to target only one biochemical reaction or pathway. But for the difficult cancers, we must think of simultaneously resetting numerous genetic pathways to produce healthier proteins. GHK may step us down a pathway to switch off cancer and turn on health.

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