NEW INSIGHT INTO CLINICAL TRIAL ON CLN IN AD – TRANSCRIPTOMAL NETWORK ANALYSIS

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ABSTRACT

Aim: Etiology of decreased cognitive functions in Alzheimer's disease (AD) is multifactorial although the most popular hypotheses are centered on the effects of the misfolded, aggregated protein, amyloid beta (Aβ) and Tau hyperphosphorylation. Recent investigations have demonstrated beneficial effect of Proline Rich Polypeptides (PRP), also known as Colostrinin™ (CLN), on cognitive symptoms and instrumental daily activities of AD patients. The aim of this study was to elucidate the mode of CLN action at molecular level.

Method: The clinical trial on 105 AD patients consisted of a 15 week double-blind phase comparing CLN with placebo, followed by a second 15 week open labeled phase when all patients received 100 µg CLN orally. The primary outcome measures were AD assessment scale-cognitive portion; clinical global impression of change; instrumental activities of daily living; and mini-mental state examination. Microarray analysis was performed using the Affymetrix GeneChip® Human Genome Focus Array containing probe sequences for well-characterized human genes verified by the NCBI RefSeq database. RNA isolation, labeling and hybridization were performed as suggested by the Affymetrix technical manual. Gene expression profiles were analyzed by Ingenuity Pathway Analysis software.

Results: The Full Sample Analysis at week 15 showed a stabilizing effect of CLN on cognitive function in ADAS-cog ($p = 0.02$) and on daily function in IADL ($p = 0.02$). The overall patient response was also in favor of CLN ($p = 0.03$). Patients graded as mild on entry also showed a superior response of ADAS-cog compared with more advanced cases ($p = 0.01$). To gain insight on how CLN stabilized/improved cognitive functions microarray analysis was undertaken. CLN elicits highly complex and multiphasic changes in 27 molecular networks that regulate important biological functions including nervous system development and function, cell proliferation, tissue/organ and immune system development and function. Importantly, transcriptomal analysis shows that CLN alters gene expression of molecular networks implicated in Aβ precursor protein (APP) synthesis, and modulates levels/activities of bleomycin hydrolase. Although tau's mRNA level was not affected, CLN decreased levels of cAMP-dependent protein kinase [PKA] and mitogen activated kinase (MKK3,6) which both regulate the phosphorylation status of mitogen-activated protein kinases (e.g., p38 MAPK) that has previously been shown to hyperphosphorylate tau. Downregulation of p38 MAPKs pathways via MKK3/6 leads to attenuation of a wide range of inflammatory cytokine-, and chemokine (e.g., IL-1, IL-6, TNF-a, TGF-β)–induced pathways that preclude neurodegeneration. CLN downregulated the expression of the main NMDA receptor (GRIN) as well as GRM7 and GRM8, G-protein coupled glutamate receptors, thus preventing supraphysiological activation of NMDA receptors and so improving neuronal plasticity. Decrease in cellular ROS levels caused by CLN prevents thiol modification and activation of redox-sensitive pathways that play an essential role in the development and progression of neurodegenerative diseases.

Discussion: This report provides possible molecular mechanisms by which CLN exerts its biological effects. Although separate studies will be needed to identify target molecules directly interacting withCLN, it appears that proline rich peptides within CLN bind to cell membrane or intracellular receptors and activate cascades of signaling leading to changes in the expression of the specific gene networks including those responsible for development and progression of AD. We conclude that CLN-mediated changes in gene expression networks might be the basis for improvement in cognitive functions of AD patients given CLN.

INTRODUCTION

A proprietary extract of proline-rich peptides from colostrum, also known as Colostrinin™ (CLN), has been shown to have a stabilizing effect on cognitive function in Alzheimer's disease (AD) patients (1,2). This complex action of CLN could be related to prevention of amyloid β peptide aggregation, as shown in *in vitro* studies, and its impact on delicate cassettes of signaling pathways common to cellular redox regulation, proliferation and differentiation (3). Studies on cultured cells showed that CLN modulates intracellular levels of reactive oxygen species (ROS), via regulating glutathione metabolism, activity of antioxidant enzymes and mitochondria function (4). Due to an improvement in senescence-associated mitochondrial dysfunction and a decrease in ROS generation, CLN decelerates the aging processes of both cultured cells and experimental animals (5). When given orally to mice, CLN increased the life-span and improved various motor and sensory activities (6). In this study we provide data for CLN-induced gene expression changes using high-density oligonucleotide arrays. CLN elicits highly complex and multiphasic changes in the gene expression profile of treated cells. CLN treatment affected a total of 58 molecular networks, 27 of which contained at least 10 differentially expressed genes. Here we present CLN-modulated gene networks as potential underlying molecular mechanisms leading to the reported effects of CLN on cellular oxidative state, chemokine and cytokine production, and cell differentiation, as well as on pathological processes like allergy, asthma, Alzheimer's, and other neurological diseases. Based on our results, we also predict possible modulatory effects of CLN on adipocytokine gene networks that play a crucial role in the pathobiology of diabetes, cardiovascular disorders, obesity, and inflammation. Taken together, CLN-altered gene expression networks presented here provide the molecular basis for previously described biological phenomena, in particular, the clinical benefit for patients with Alzheimer's disease.

CLINICAL TRIALS

Study Design/Patient Population/Dose

A multicenter trial was undertaken, consisting of placebo/controlled, double-blind treatment for 15 weeks (Phase I), followed by a 15 weeks open – label phase during which all patients received active therapy (Phase 2) as described earlier (2). Phase 1 was designed to evaluate the efficacy of CLN compared with placebo in mild to moderately severe cases of probable AD. Phase 2 looked at long term tolerability and sustained efficacy of CLN. Eligible patients were 50 years of age and older suffering from mild AD as defined in the fourth edition of the Diagnostic and Statistical Manual 1V (DSM-1V) and National Institute of Neurological and Communicative Disorders and Stroke, AD, and Related Disorders Association (NINCDS-ADRDA). Only mildly or moderately affected patients were admitted to the study as measured on the MMSE i.e. 10 to 24 points inclusive. Only ambulatory patients able to attend the clinic accompanied by a relative or caregiver were enlisted, either of whom could provide written, informed consent. Patients with severe somatic disorders, especially those likely to be associated with dementia, e.g. cerebrovascular disease (Hachinski ischaemic score > 6), endocrine disorders, Parkinson's Disease, psychoses, schizophrenia and severe affective disorders, were excluded. Evidence of neurological abnormalities on recent brain scans (other than those changes expected with probable AD) excluded the patient from the trial.

The CLN treatment group received tablets containing 100 µg of active substance plus excipients (mannitol, magnesium stearate and sodium chloride). The placebo patients received identical tablets containing no active ingredient. Patients allocated to the CLN group received one active tablet on alternate days (i.e days 1, 3, 5 etc.) interspersed with a placebo tablet on the even days. This regimen was administered for three weeks followed by two weeks treatment of daily placebo only. Patients in the control group received one placebo tablet daily throughout the five week period. The complete five week cycle was repeated three times throughout Phase 1. Patients who completed the double-blind Phase 1 proceeded to Phase 2 where they were all treated with CLN using the same dosage regimen as in the CLN group of Phase 1 but in open label.

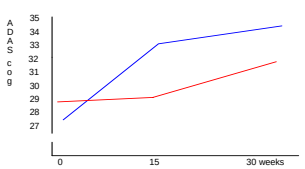

FIGURE 1. Alzheimer's Disease Assessment Scale Cognitive Subscale (ADAS-cog) changes in median values over 30 weeks. Group 1: Red, active/active; Group 2: Blue, placebo/active. ADAS-cog is scored by errors (with a total error score range of 0 to 70). ADAS cog; p=0.02.

Clinical Results

Results showed that CLN was significantly better than placebo in some but not all tests. Statistically significant differences ($p = 0.02$) were found in the primary efficacy parameter ADAS-cog and in the secondary variable Instrumental Activities of Daily Living (IADL) on the FSA. Thus, the effect on two major efficacy parameters (one measuring cognitive changes and the other functional changes in daily activities) proved favorable. The overall benefit analysis in the full sample showed that 40% of all patients stabilized or improved on CLN at week 15 as opposed to only 21% on placebo. This figure reduced to 33% on CLN at week 30 (Group 1) but showed a stabilization of Group 2 when converted to CLN during Phase 2. Finally a subset analysis showed that patients stratified as mild on entry responded much better ($p = 0.01$, FSA) than more advanced cases. This differential response between mild and moderate cases was maintained throughout the study and may be considered an important therapeutic finding if confirmed in future studies. The incidence and nature of adverse events reported were similar in both the active and placebo groups. Generally, the treatment was well tolerated and the relatively high drop-out rates due to side-effects associated with the ChEI were not encountered in this study. Most importantly, this statistically significant improvement in cognitive functions of AD patients has been accomplished with a low dose CLN regimen.

To gain insight into the molecular mechanisms by which low dose CLN exerts its complex biological effects in AD patients treated orally, we performed microarray analysis of RNA isolated from CLN-treated cells.

EXPERIMENTAL DESIGN/CELL CULTURE AND TREATMENT

The TR146, human buccal mucosal cell line was obtained from the Cancer Research Institute, London, England. Cells were propagated in high glucose, Dulbecco's modified Eagle medium supplemented with 3.7 mg/ml NaHCO3, 10% FCS, 50 units/ml penicillin-G, and 50 µg/ml streptomycin. Cells were subcultured when they reached 90% confluence. Unless otherwise stated, all tissue culture related materials were purchased from Gibco/Invitrogen, Carlsbad, CA. For microarray analysis, cells at 75-80% confluence in T75 flasks (Corning, Lowell, MA) were treated with 100 ng/ml Colostrinin™ of bovine origin (ReGen Therapeutics Plc, London, UK). After 3 hours the cells were washed twice with DPBS, trypsinized, suspended in 5 ml growth media, and centrifuged (800 x g for 10 min). Cell pellets were used for RNA isolation.

Microarray analysis was performed using the Affymetrix GeneChip® Human Genome Focus Array containing probe sequences for well-characterized human genes verified by the NCBI RefSeq database. The labeling and hybridization protocols were performed as suggested by the Affymetrix technical support manual.

Molecular network and pathway analysis identified the gene networks and canonical pathways from the Ingenuity Pathways Analysis library that were most significant to the dataset. The significance of the association between the dataset and the canonical pathway was measured in 2 ways: 1) a ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway was calculated; and, 2) Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway could not be explained by chance alone. A cutoff p-value of $p \leq 0.05$ was used to determine significantly affected networks.

RESULTS

Gene Expression Results

The goal of this study was to elucidate the mechanism of action by which CLN, given orally, showed therapeutic effect. It was hypothesized that CLN can induce signaling cascades to modulate various metabolic processes (7). Here we demonstrate that CLN elicits highly complex and multiphasic changes in the gene expression profile of treated cells. A total of 58 molecular networks, 27 of which contained at least 10 differentially expressed genes have been affected. Each molecular network was further analyzed to identify the biological functions and/or diseases that were most significant to that network according to the IPA Knowledge Base.

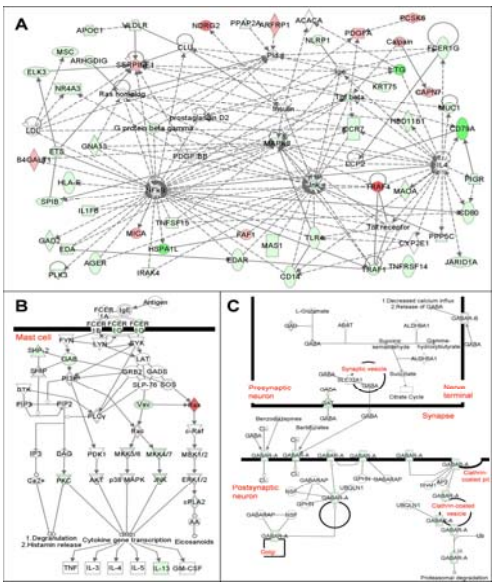

FIGURE 2. CLN affects immune functions and inflammation related gene networks**. A.** Immune system development and functions; **B.** Fc epsilon receptor signaling; **C.** GABAA receptor signaling.

CLN effects on immune system function and associated disease networks

Our network analysis identified significant CLN-induced alterations in gene networks associated with immune system development and function (Fig. 2). Seven out of 27 top-scoring networks were identified as being involved in these processes. As shown in Fig. 2A CLN downregulated 46 out of 66 genes involved in the regulation of inflammatory pathways, including tumor necrosis factor family genes, mitogen-activated protein kinases (MAPK) and c-Jun N-terminal kinase (JNK), that are key members of the stress kinase pathway and modulators of inflammatory processes. Fig. 2c illustrates consistent downregulation of GAD and GABAARs in epithelial cells. These data strongly suggest that this could be the molecular mechanism by which CLN prevents development of inflammation.

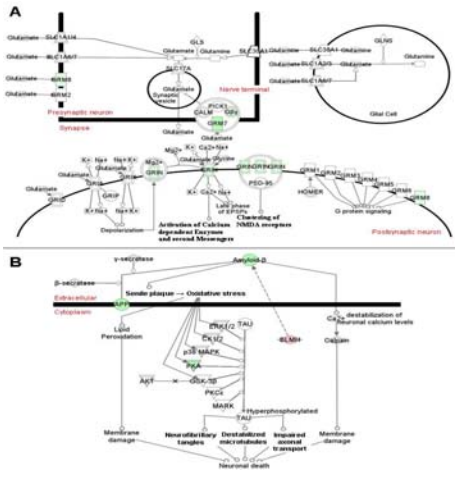

FIGURE 3. CLN downregulates glutamate receptor signaling and amyloid beta producing pathways. A. Glutamate receptor signaling; B. Amyloid beta processing.

CLN effects on nervous system function and associated disease networks

CLN downregulated the expression of the main NMDA receptor (GRIN) as well as GRM7 and GRM8, G-protein coupled glutamate receptors as shown in Figure 3A. This mechanism of preventing supraphysiological activation of NMDA receptors and improving neuronal plasticity might contribute to improved cognitive functions in AD patients after CLN treatment. Hypotheses regarding decreases in cognitive functions in Alzheimer's disease are centered on the effects of the misfolded, aggregated protein, amyloid beta (Aβ) and Tau hyperphosphorylation. Figure 3B illustrates CLN effects on the canonical pathway of Aβ processing. CLN downregulates Aβ precursor protein (APP) mRNA levels thereby potentially decreasing levels of aggregated Aβ, which has convincing implications in Alzheimer's disease. Central to the amyloid hypothesis of Alzheimer's disease is an altered proteolytic processing of the Aβ precursor protein (APP) by α-, β -, and γ-secretases resulting in overproduction and aggregation of neurotoxic forms of Aβ. CLN's reported beneficial effect on cognitive functions in Alzheimer's disease may be due to its ability to suppress APP gene expression and modulate cellular redox. In addition, it has been shown that bleomycin hydrolase cleaves Aβ-peptides including Aβ(1-40); Aβ(1-42) and Aβ(3-42). It is remarkable that CLN upregulates bleomycin hydrolase at the mRNA level (Figure 2B). Therefore, upregulation of bleomycin hydrolase together with downregulation of APP expression results in decreased Aβ accumulation. Tau hyperphosphorylation is another hallmark of AD. In neurons affected by tauopathy (e.g., AD, Guam parkinsonism dementia), hyperphosphorylated tau is found not only in axons but also in cell bodies and dendrites as well as extracellularly. Not surprisingly, tau's mRNA level is not affected by CLN treatment (Figure 3B), because its function is altered by post-translational modification. However, tau was shown to be hyper-phosphorylated by protein kinases, including cyclic AMP-dependent protein kinase (PKA) calcium, calmodulin-dependent protein kinase II (CK1/2), mitogen-activated protein kinases (ERK1/2, MEK1/2 and p38 MAPK), glycogen synthase kinase-3β (GSK-3β), and protein kinase Cε (PKCε). The network in Figure 3B shows that CLN treatment downregulated PKA (one of the rate-limiting kinases in Tau phosphorylation) expression. Finally, it was remarkable that CLN up-regulated two genes encoding for the key antioxidant enzymes glutathione-disulfidreductase and transporter of glutathione (GSR) through the sarcoplasmic reticulum, although these cells were not challenged oxidatively (Fig 4). These data support the notion that CLN increases cellular stress tolerance via regulating glutathione (GSH) metabolism.

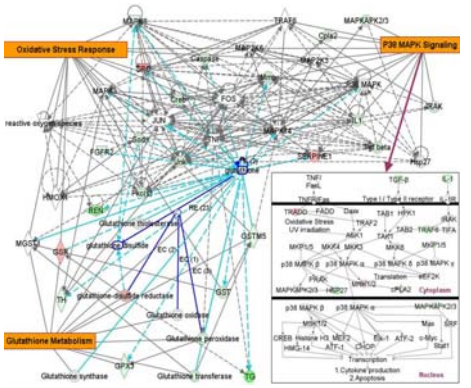

FIGURE 4. Oxidative stress related molecular networks affected by Colostrinin. Immediate interactions of glutathione and glutathione-disulfide are represented by blue edges. Insert: Canonical pathway of p38 MAPK signaling overlaid by the gene expression level changes after colostrinin treatment EC: Enzyme Catalysis; E: Expression; RE: Reaction.

CONCLUSIONS

Oxidative stress is believed to be one of the leading contributors to aging processes and to age-associated diseases, such as Alzheimer's disease, the most frequent form of dementia among the elderly. Oxidative damage occurs early in the brain of Alzheimer's disease patients, before the onset of plaque pathology, and is characterized by such neuropathological hallmarks as extracellular Aβ plaques and intracellular neurofibrillary tangles, composed of the abnormally hyperphosphorylated protein, tau. Tau and Aβ, as well as mitochondrial dysfunction, exhibit synergistic effects leading to deficient energy metabolism and accelerated neurodegeneration. When given orally, CLN has been shown to have a stabilizing effect on cognitive functions in improving the conditions of patients suffering from mild and moderate AD. Peptides in CLN bind to as-yet-unidentified membrane receptor(s), and induce signaling pathways that appear to be common to regulation of cell proliferation, differentiation and amelioration of chemokine/cytokine production in inflammatory processes (Fig 5). This is the first report which provides possible molecular mechanisms by which CLN exerts its biological effects and perhaps explains why CLN can achieve these effects at such low dose levels.

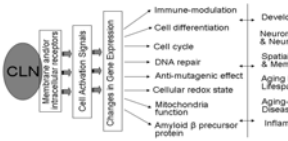

FIGURE 5. CLN potential Mode of Action. Signal transduction/potentiation effects as evident in clinical trials on AD

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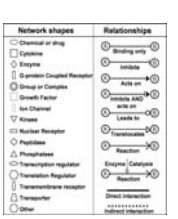

Node shapes and edge types in networks and pathways generated by Ingenuity Pathway Analysis.

For signaling pathways an arrow pointing from A to B signifies that A causes B to be activated. For metabolic pathways an arrow pointing from A to B signifies that B is produced from A. For ligands/receptors an arrow pointing from a ligand to a receptor signifies that the ligand binds the receptor and subsequently leads to activation of the receptor. "Acts on" and "Inhibits" edges may also include a binding even.

The nodes are coloured red to indicate upregulation, green to indicate downregulation and gray to indicate nodes which did not reach the threshold for expression level (≥200 signal) and/or for fold change (≥2x).

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