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NEW INSIGHTS INTO CLINICAL TRIAL FOR COLOSTRININ™ IN ALZHEIMER’S DISEASE

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Abstract: Background: The pathomechanism of 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 on Tau hyperphosphorylation. Objectives: Double blinded clinical trials were planned to demonstrate the effect of Colostrinin™ (CLN) on instrumental daily activities of AD patients. The potential molecular mechanisms by which CLN mediates its effects were investigated by gene expression profiling. Methods: RNAs isolated from CLN-treated cells were analyzed by high-density oligonucleotide arrays. Network and pathway analyses were performed using the 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 to more advanced cases (p = 0.01). Data derived from microarray network analysis show that CLN elicits highly complex and multiphasic changes in the cells’ transcriptome. Importantly, transcriptional analysis showed that CLN alters gene expression of molecular networks implicated in Aβ precursor protein synthesis, Tau phosphorylation and increased levels of enzymes that proteolytically eliminate Aβ. In addition, CLN enhanced the defense against oxidative stress and decreased expression of inflammatory chemokines and cytokines, thereby attenuated inflammatory processes that precede Alzheimer’s and other neurological diseases. Conclusion: Together these data suggest that CLN has promising potential for clinical use in prevention and therapy of Alzheimer’s and other age-associated central nervous system disease.

Key words: AD, CLN, network analysis.

Introduction

Alzheimer’s disease (AD) is the sole cause of about half of the cases of dementia in later life and a significant contributor to cognitive decline in a further quarter. It is therefore by far the most important factor in what has been called the silent epidemic of dementia that is occurring in societies with aging populations. Since the incidence of AD can be expected to rise in all societies where economic progress leads to increased life expectancy, AD along with tuberculosis and malaria has gained economic and social importance. In part for this reason, a significant amount of research has addressed AD in the last decades, and much progress has been made in our understanding of AD pathophysiology.

Currently various hypotheses exist for the pathogenesis of AD. Among these the acetylcholine- (1), the amyloid-cascade- (2-4), and the "inflammation of the brain-" (5) hypotheses are the most established. Recently the oxidative-stress-hypothesis has evolved, integrating and combining significant portions of these hypotheses. It is now evident that due to their extreme reactivity, free-radicals may cause molecular damage, changes in cell activation signals and cell death in various tissues, including the central nervous system (6, 7).

A proprietary extract of proline-rich peptides, also known as Colostrinin™ , has been shown to have a stabilizing effect on cognitive function in Alzheimer’s disease (8-10). CLN complex action could be related to prevention of Aβ peptide aggregation, as shown in in vitro studies (11), and its impact on signaling pathways common to cellular redox regulation, proliferation, and differentiation (7, 12, 13). Studies on cultured cells showed that CLN modulates intracellular levels of reactive oxygen species (ROS), via regulating glutathione metabolism, the activity of antioxidant enzymes, and mitochondrial function (12, 14). Due to an improvement in senescence-associated mitochondrial dysfunction and a decrease in ROS generation, CLN decelerates aging processes of both cultured cells and experimental animals (12, 15). When given orally to mice, CLN increased the life-span and improved various motor and sensory activities (15-17).

Here we report a stabilizing effect of CLN on cognitive functions in Alzheimer’s disease patients. In addition, evidence are provided for CLN-mediated highly complex and multiphasic cellular changes in gene expression. The extensive changes in the CLN-modulated gene networks provide a hint of the underlying molecular mechanisms by which CLN may exerts its beneficial effect in Alzheimer’s patients.

Materials and methods

Clinical Study Design

A multicenter trial was undertaken, consisting of placebo-controlled, double-blind treatment for 15 weeks (Phase 1), followed by a 15 weeks open – label phase during which all patients received active therapy (Phase 2) as described earlier.
(8). Phase 1 was designed to evaluate the efficacy of CLN compared with placebo in 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 IV (DSM-IV) 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.

**Cell cultures 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. 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 ColostrininTM of bovine origin (ReGen Therapeutics Plc, London, UK). At various times (3, 6, 12 and 18 hours) after CLN addition cells were washed twice with Dulbecco-modified PBS, trypsinized, and centrifuged (800 x g for 10 min). Cell pellets were used for RNA isolation.

**RNA extraction, cDNA and cRNA synthesis**

Total RNA was isolated using Ambion’s RNAquous Kit (Ambion, Austin, TX) as recommended by the manufacturer. Total RNA concentration was quantified using a DU530 Beckman spectrophotometer (Beckman Coulter, Fullerton, CA) at 260 nm. The integrity of total RNA samples was assessed qualitatively using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). First and second strand cDNA syntheses were performed using Superscript Choice System for cDNA Synthesis kit (Invitrogen, Carlsbad, CA). In vitro transcription reactions to produce biotin-labeled cRNA from the cDNA were performed using Affymetrix RNA Transcript Labeling Kit. Biotin-labeled cRNA was then purified and fragmented using the GeneChip Sample Cleanup Module.

**Microarray analysis**

Microarray analysis was performed using the Affymetrix GeneChip® Human Genome Focus Array. Labeling and hybridization protocols were performed as directed by the Affymetrix technical support manual (http://www.affymetrix.com/support/technical/manual/expression_manual.affx). Hybridization of the cRNA target onto GeneChip Human Genome Focus Arrays (Affymetrix Inc., Santa Clara, CA) was carried out in an Affymetrix Hybridization Oven-640 at 45°C at a rotation speed of 60 rpm. After staining with phycoerythrin-conjugated streptavidin (Molecular Probes/Invitrogen, Carlsbad, CA), gene-chips were scanned by Affymetrix GeneChip Scanner 3000 7G at 570 nm wavelength after 530 nm excitation. The resulting image file was saved and analyzed by Affymetrix Microarray Suite software, as we previously described [18].

**Analysis of data**

Changes in gene expression were analyzed by Microsoft Excel 2003 software (Microsoft, Redmond, CA), the Affymetrix NetAFFX Analysis Center online tools, and Spotfire Decision Site 9.0 software (TIBCO Spotfire, Somerville, MA). Expression levels for genes of CLN-treated samples were compared to the corresponding gene expression levels from the untreated controls in a pair-wise fashion. Genes with increased or decreased expression levels (≥2-fold difference compared to control) were selected for further analysis. A threshold of 200 signal intensity units was used for detectability.

**Ingenuity Pathway Analysis**

Network and pathways analyses were performed using the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Inc., Redwood City, CA; www.ingenuity.com). Briefly, each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. Genes with 2-fold change in expression called focus genes, were overlaid onto a global molecular network in the Ingenuity Pathways Knowledge Base. These networks were ranked based on the significance score value calculated by the IPA software. A graphical representation of the molecular relationships between genes/gene products for each of the top ranked networks was generated where genes or gene products are
represented as nodes, and the biological relationship between two nodes is represented as an edge (line). The nodes are colored red to indicate up-regulation, green to indicate down-regulation and gray to indicate genes which did not reach the threshold for expression level ($\geq 200$ signal) and/or for fold change ($\geq 2x$). The intensity of the color of the node corresponds to the degree of change in gene expression. Different node shapes and edges represent different molecule types and relationships (Fig. 1).

**Figure 1**
Node shapes and edge types in networks and pathways generated by Ingenuity Pathway Analysis

An arrow pointing from A gene to B gene signifies that A causes B to be activated. For metabolic pathways an arrow pointing from A to B shows 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 event.

**Statistical Analysis**
The significance of the association between the dataset and the canonical pathway was determined by the following methods: 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 p-values. A cutoff p-value of p<0.05 was used to identify significantly affected networks.

**Results and discussion**

**Clinical Study Results**
Results from the multicenter clinical trials consisting of placebo/controlled, double-blind treatments showed that CLN was significantly better than placebo in some but not all tests (Figure 2). Statistically significant differences ($p = 0.02$) were found in the primary efficacy parameter (Alzheimer’s disease assessment scale-cognitive) and in the secondary variable instrumental activities of daily living. 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 AD 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. CLN treatment was well tolerated and drop-outs due to minor side-effects 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 (8-10).

**Figure 2**
Alzheimer’s Disease Assessment Scale Cognitive Subscale (ADAS-cog) changes in median values over 30 weeks

Red: Group 1, active/active; Blue: Group 2, placebo/active. ADAS-cog is scored by errors (with a total error score range of 0 to 70). ADAS-cog: $p=0.02$. (Bilikiewicz et. al. 2004)

Overall changes in Gene Expression
To gain insight into the molecular mechanisms by which CLN may exert its complex biological effects in AD patients, we performed microarray analysis of RNA isolated from CLN-treated cells. We found 450, 27, 107, and 318 differentially expressed genes at 3, 6, 12, 18 hours after treatment respectively. At 3 h time point, 271 genes were up- and 179 down-regulated. Most of the initially responding genes returned to basal levels by 6 h after treatment. Secondary responses were initiated from 6 and 12 h on. At 18 h, 185 genes were down- and 133 genes were up-regulated. Although we have extensive amounts of data for late (6, 12 and 18 h) cellular responses, we considered them as multilayered changes in gene expression and as a consequence of the primary CLN
effect, therefore they are not presented here. Differentially expressed genes induced by CLN at 3 h were selected for transcriptome analysis. The 450 differentially expressed genes belong to 58 well defined molecular networks, 27 of which contained at least 10 differentially expressed genes (19). 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 (19).

**CLN-mediated changes in inflammatory pathways**

Inflammation is the body’s (acute or chronic) response to pathogens, chemical, or physical injury. In contrast to acute inflammation, prolonged inflammation is a dysregulated response of tissues that involves active inflammation and tissue destruction. Such persistent inflammation is associated with many chronic human diseases, including cardiovascular and autoimmune diseases; and it precedes the development of diseases of the central nervous system including Alzheimers’ disease.

As we have shown previously (19) CLN down-regulates interleukin (IL) receptor type I (IL RI). Upon binding of IL-1 (e.g., IL-1β) to IL-1 RI, it becomes heterodimerized with the IL-1 RI-associated protein, then IL-1 RI-associated kinases (e.g., IRAK-1, -2, and -4) are recruited (20). IL-1R1 activation signaling leads to a large number of cellular changes culminating in inflammatory processes. IL-1 β is secreted by many cell types in the haemopoetic system (e.g., monocytes and macrophages). IL-18 enters the circulation and triggers IL-1 receptors on several cells types including cells in the hypothalamic vascular network, bone marrow and endothelium. IL-1 triggers production of IL-6 and other cytokines. IL-6 stimulates liver hepatocytes to synthesize several acute phase proteins, but most importantly its secretion by peripheral blood mononuclear cells was increased in patients with AD as reviewed by Ravaglia et al., 2007 (21). In addition, signals by mononuclear cells was increased in patients with AD as reviewed by Ravaglia et al., 2007 (21). In addition, signals by mononuclear cells was increased in patients with AD as reviewed by Ravaglia et al., 2007 (21). In addition, signals by mononuclear cells was increased in patients with AD as reviewed by Ravaglia et al., 2007 (21).

**Figure 3**

CLN affects inflammation-related gene networks

![Figure 3](image-url)
regulation of resistin and IL-1 signaling by CLN is an important finding and may constitute to the beneficial effect of CLN in AD patients. Our data strongly suggest that this could be a key mechanism by which CLN prevents development of IL-1, IL-6-mediated inflammation that often precedes AD, and other age-associated central nervous system diseases.

CLN effects on networks implicated in nervous system function and diseases

In the nervous system, the main excitatory neurotransmitter is the glutamate, which mediates its effects via ionotropic and metabotropic receptors. The primary glutamate receptor is ionotropic and specifically sensitive to N-Methyl-D-Aspartate (NMDA) (34). Perturbation of glutamatergic neurotransmission is involved in the pathogenesis of neurodegenerative diseases including Alzheimer’s and Huntington’s disease, amyotrophic lateral sclerosis, the propagation of seizure activity in epilepsy, and post-stroke neurodegeneration (1, 35-38). CLN down-regulated the expression of the main NMDA receptor (GRIN) as well as GRM7, GRM8, G-protein coupled glutamate receptors as shown in Fig. 5. If this effect will be proven in vivo, this could be one of the mechanisms by which CLN prevents supra-physiological activation of glutamate receptors and improves neuronal plasticity and cognitive functions in AD patients.

Our previous findings show that CLN treatment down-regulates mRNA levels for resistin and alters the expression genes in resistin-induced networks (19). It has been shown that resistin is engaged in inflammatory conditions in humans through IL-1, IL-6 and TNF-α (31, 32).

Experimental evidence shows that resistin induces and becomes induced by IL-1, IL-6 and TNF-α (all via the NF-kB pathway). It should be pressed that Aβ peptide production itself can induce increased expression of IL-1β, TNF-α, and IL-6 in astrocytes and microglial cells in culture. IL-1 and IL-6 has been found to be important in early stages of senile plaque formation (33). It was also observed that IL-6 secretion by peripheral blood mononuclear cells was increased in patients with AD as opposed to normal subjects or those suffering from other brain disorders such as vascular dementia (21). Studies in IL-6 overexpressing mice showed that this cytokine is associated with a variety of neuropathological findings, including tauopathy(s), gliosis and disruption of cholinergic neurotransmission in the hippocampus (33). Therefore, down-regulation of resistin and IL-1 signaling by CLN is an important finding and may constitute to the beneficial effect of CLN in AD patients. Our data strongly suggest that this could be a key mechanism by which CLN prevents development of IL-1, IL-6-mediated inflammation that often precedes AD, and other age-associated central nervous system diseases.

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and Tau hyperphosphorylation. Fig. 6 shows potential effects of CLN on the canonical pathways of Aβ processing. First, CLN down-regulates Aβ precursor protein (APP) mRNA levels thereby potentially decreasing levels of aggregated Aβ. Second, central to the formation of amyloid plaques is the altered proteolytic processing of the APP by α-, β-, and γ-secretases. Although it did not reach statistical significance, CLN decreased expression of β- and γ-secretases (Fig. 6). Third, it has been shown that bleomycin hydrolase (BLMH) cleaves Aβ-peptides including Aβ(1-40); Aβ(1-42) and Aβ(3-42). It is remarkable that CLN up-regulates BLMH at the mRNA level (Fig. 6). Therefore, upregulation of BLMH together with downregulation of APP and β-secretase expression may results in decreased Aβ accumulation in vivo.

**Figure 6**

Effects of CLN on amyloid beta processing and Tau hyperphosphorylation


**Conclusion**

Among age-associated diseases of the central nervous system, Alzheimer's disease is the most frequent form of dementia in elderly. Orally administered CLN showed significant stabilizing effect on cognitive functions in improving the conditions of patients suffering from mild and moderate AD. The molecular mechanisms, by which CLN exerts its effects are not known. Data presented here suggest that peptides in CLN induce signaling pathways that result in changes in molecular networks regulating stress kinase pathways, inflammatory cytokine and chemokine production. Importantly, transcriptomal analysis shows that CLN alters gene expression of molecular networks directly implicated in Aβ precursor protein synthesis, and increases levels of an Aβ-hydrolyzing enzyme. Although Tau’s mRNA level was not significantly affected, CLN decreased levels of cAMP-dependent protein kinase A and mitogen activated kinases kinase 3 and 6, which all participate in the hyperphosphorylation Tau. Downregulation of p38 mitogen activated kinase pathways via upstream kinases 3 and 6 leads to attenuation of a wide range of inflammatory cytokine-, and chemokine–induced pathways that precede neurodegeneration. CLN modulates the expression of the main glutamate receptors, as well as GABA signaling, thus it has the potential to dampen symptoms associated with AD and other central nervous system diseases. Taken together, these data suggest that CLN has potential clinical benefit in prevention and treatment of various age-associated neurological diseases.

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