The Journal of Nutrition, Health & Aging©
Volume , Number , 2009

THE JOURNAL OF NUTRITION, HEALTH & AGING©

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

P. SZANISZLO[1,3], P. GERMAN[1,3], G. HAJAS[1,3], D.N. SAENZ[1,3], M. KRUZEL[2], I. BOLDOGH[1,4]

1. Department of Microbiology and Immunology, UTMB Galveston, TX, USA; 2. PharmaReview Corporation, Houston, Texas; 3. These authors contributed equally to this work; 4. Corresponding author: Istvan Boldogh, D.M&B, Ph.D; Department of Microbiology and Immunology; University of Texas Medical Branch at Galveston; 301 University Blvd, Galveston, Texas 77555, Telephone: 409-772-9414, Fax: 409-747-6869; Email: (sboldogh@utmb.edu).

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, transcriptomal analysis showed that CLN alters gene expression of molecular networks implicated in Aβ precursor protein synthesis, Tau phosphorylation and increased levels of enzymes that proteolitically 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 global 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' 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 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.

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 RNAqueous 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 detectibility.

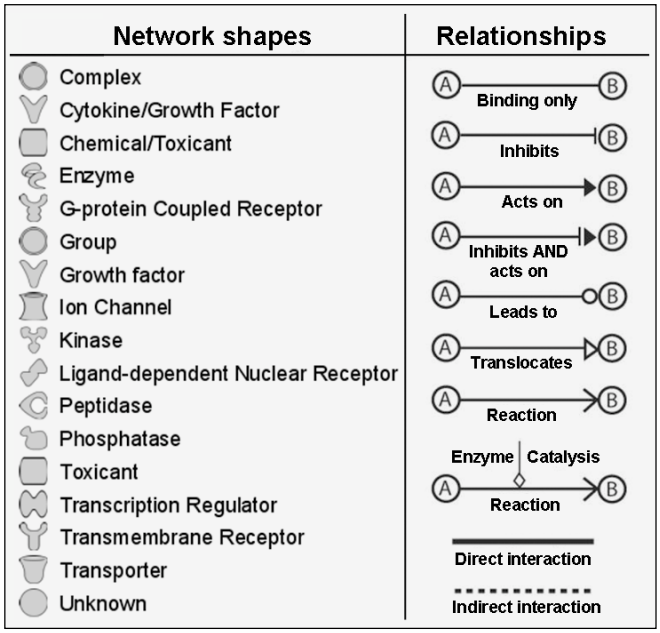
Ingenuity Pathway Analysis

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 (≥200 signal) and/or for fold change (≥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

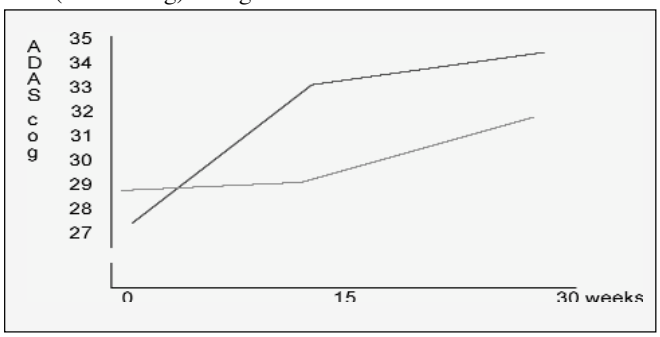
The significance of the association between the dataset and the canonical pathway was determined by the following methods: 1) a ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway was calculated; and 2) Fisher's 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

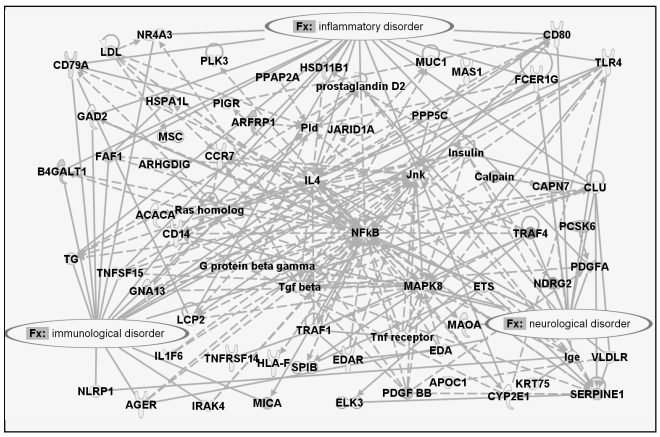
Inflammation is the body's (acute or chronic) response to pathogens, chemical, or physical injury. In contrast to acute inflammation, prolonged inflammation is a dysregulated response of tissues that involves active inflammation and tissue destruction. Such persistent inflammation is associated with 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-1RI activated signaling leads to a large number of cellular changes culminating in inflammatory processes. IL-1 β is secreted by many cell types in the haemapoetic system (e.g., monocytes and macrophages). IL-1ß 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 TNF-α receptor-associated factor-6 (TRAF-6) were down-regulated by CLN; they otherwise would lead to the activation of nuclear factor kappa B (NF-ϰB). NF-ϰB is a family of seven structurally related transcription factors that play a central role in inflammation by controlling the expression of inflammatory gene networks (22, 23).

In the "transcriptomic snapshot" shown in Fig. 3 CLN down-regulated 46 out of 66 genes involved in the regulation of inflammatory pathways including tumor necrosis factor (TNF) family (e.g., TNFSF 14 and 15) 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 (24, 25). In addition, as shown in Fig. 4a, CLN also alters the canonical pathways of Fc epsilon receptor signaling, which has a role in the activation of cells, specifically mast cells (first-line producers of inflammatory cytokines and chemokines, e.g., TNF-α, IL-4, IL5, IL-13) in response to antigenic stimuli (26). CLN down-regulates the expression of the gamma subunit of the Fc epsilon receptor, JNK, protein kinase C (PKC), phospholipase A2 (cPLA2) and interleukin 13 (IL-13) expression. These data are in line with the findings that CLN significantly decreased levels of allergen-induced IgE/IgG1, mucin levels, and lung inflammation (27). Fig. 4b illustrates the effects of CLN on the canonical pathway of gamma-aminobutyric acid (GABA) receptor signaling. CLN down-regulates expression of glutamic acid decarboxylase (GAD) and GABA-A receptor (GABAAR). GABA is an important neurotransmitter that, among many other functions, regulates mucus production in airway epithelial cells through the subtype A GABAAR (28, 29). The GABA system is the major signal transmitter in the human brain and it was found to be associated with pathophysiology of epilepsy, anxiety, aggression, and psychosis as reviewed by Lanctot et. al., 2004 (30). Importantly GABA has been shown to be potential therapeutic target for controlling AD symptoms (30).

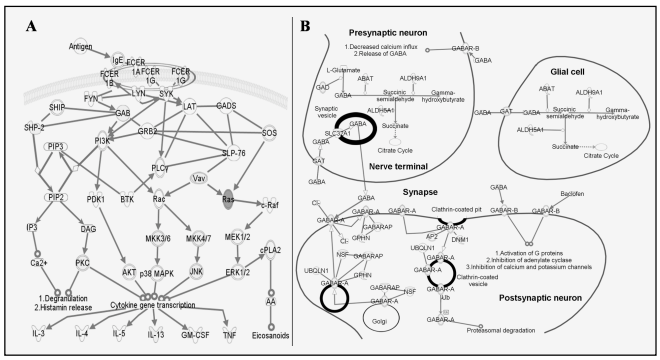
Figure 3

CLN affects inflammation-related gene networks



ACACA:acetyl-Coenzyme A carboxylase alpha, AGER:advanced glycosylation end product-specific receptor, APOC1:apolipoprotein C-I, ARFRP1:ADP-ribosylation factor related protein 1, ARHGDIG:Rho GDP dissociation inhibitor gamma, B4GALT1:beta 1,4-galactosyltransferase, CAPN7:calpain 7, CCR7:chemokine receptor 7, CD14:CD14 antigen, CD79A:immunoglobulin-associated alpha, CD80:CD80 antigen, CLU:clusterin, CYP2E1:cytochrome P450 2/E/1, DAG:diacylglycerides, EDA:ectodysplasin A, EDAR:ectodysplasin A receptor, ELK3:SRF accessory protein 2, ETS:v-ets E26 oncogene homolog, Ige:immunoglobulin heavy constant epsilon, IgE:immonoglobulin E, IL:interleukin, IP3:inositol 1,4,5-trisphosphate, IRAK4:interleukin-1 receptor-associated kinase 4, JARID1A:jumonji, AT rich interactive domain 1A, JNK:Jun kinase, KRT75:keratin 75, LCP2:lymphocyte cytosolic protein 2, LDL:low density lipoprotein, MAOA:monoamine oxidase A, MAPK8:mitogen-activated protein kinase 8, MAS1:MAS1 oncogene, MEK1/2:MAP kinase kinase 1/2, MICA:MHC class I polypeptide-related sequence A, MSC:musculin, MUC1:mucin 1, NDRG2:n-myc downregulated gene 2, NFkB:nuclear factor kappa B, NLRP1:NLR pyrin domain containing 1, NR4A3:nuclear receptor 4/A/3, p38 MAPK:p38 MAP kinase protein, PCSK6:proprotein convertase subtilisin 6, PDGF BB:platelet-derived growth factor b dimer, PDGFA:platelet-derived growth factor alpha, PDK1:3-phosphoinositide dependent protein kinase-1, PI3K:phosphoinositide-3-kinase, PIGR:polymeric immunoglobulin receptor, PKC:protein kinase C, Pld:phospholipase D, PLK3:polo-like kinase 3, PPAP2A:phosphatidic acid phosphatase 2A, PPP5C:protein phosphatase 5, SERPINE1:serpin peptidase inhibitor E1, SHIP:inositol polyphosphate-5 phosphatase, SHP-2:protein tyrosine phosphatase 11, SLC32A1:solute carrier 32 A1, , SPIB:Spi-B transcription factor, SYK:spleen tyrosine kinase, TG:thyroglobulin, Tgf beta:transforming growth factor beta, TLR4:toll-like receptor 4, TNF:tumor necrosis factor, Tnf receptor:tumor necrosis factor receptor, TNFRSF:tumor necrosis factor receptors, TRAF1:TNF receptor-associated factor 1, TRAF4:TNF receptor-associated factor 4, Ub:polyubiquitin, VLDLR:very low density lipoprotein receptor. Network node shapes, colors, and edge types as in Fig.1. 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).

Figure 4
CLN-mediated alterations in Fc epsilon receptor (A) and GABAA receptor (B) signaling



A, AA:arachidonic acid, AKT:akt viral oncogene homolog, BTK:Bruton agammaglobulinemia tyrosine kinase, cPLA2:phospholipase A2, c-Raf:raf viral oncogene homolog, DAG:diacylglycerides, ERK1/2:MAP kinase 1/3, FAF1:Fas associated factor 1, FCER1A/B/G:Fc epsilon receptor alpha/beta/gamma, FCER1G:Fc epsilon receptor 1 gamma, FYN:FYN oncogene, GAB:GRB2-associated binding protein 1, GRB2:growth factor receptor-bound protein 2, GADS:GRB2-related adaptor protein 2, IP3:inositol 1,4,5-trisphosphate, JNK:Jun kinase, LAT:linker for activation of T cells, LYN:LYN viral oncogene homolog, MAS1:MAS1 oncogene, MEK1/2:MAP kinase kinase 1/2, MICA:MHC class I polypeptide-related sequence A, MKK3/6:MAP kinase kinase 3/6, MKK4/7:MAP kinase kinase 4/7, :3-phosphoinositide dependent protein kinase-1, PI3K:phosphoinositide-3-kinase, PLC-gamma:phospholipase C gamma, PDK1:3-phosphoinositide dependent protein kinase-1, PIP2:1-phosphatidyl-1D-myo-inositol 4,5-bisphosphate, PIP3:phosphatidyl-inositol 3,4,5-trisphosphate, PKC:protein kinase C, Rac:ras-related small G-protein, Ras:ras viral oncogene homolog, Ras homolog:Rho GTPase, SHP:protein tyrosine phosphatase 11, SYK:spleen tyrosine kinase, SLP-76:lymphocyte cytosolic protein 2, SOS:son of sevenless, Vav:vav guanine nucleotide exchange factors, TNF:tumor necrosis factor receptors. **B**, ABAT:4-aminobutyrate aminotransferase, ALDH5A1:aldehyde dehydrogenase 5A1, ALDH9A1: aldehyde dehydrogenase 9A1, AP2:adaptor protein 2, DNM1:dynamin 1, GAB:GRB2-associated binding protein 1, GABA:gamma-aminobutyric acid, GABAR-A:GABA A receptor, GABARAP:GABA A receptor-associated protein, GABAR-B:GABA B receptor, GAD:glutamate decarboxylase, GAD2:glutamate decarboxylase 2, GADS:GRB2-related adaptor protein 2, GAT:GABA transporter, GM-CSF:colony stimulating factor 2, GNA13:G-protein alpha 13, GPHN:gephyrin, GRB2:growth factor receptor-bound protein 2, HLA-F:major histocompatibility complex IF, HSD11B1:hydroxysteroid (11-beta) dehydrogenase 1, HSPA1L:heat shock 70kDa protein 1-like, NSF:N-ethylmaleimide-sensitive factor, UBQLN1:ubiquilin 1. In A and B, network node shapes, colors, and edge types as in Fig. 1. The nodes are coloured red to indicate up-regulation, green to indicate down-regulation and gray to indicate nodes which did not reach the threshold for expression level (≥200 signal) and/or for fold change (≥2x).

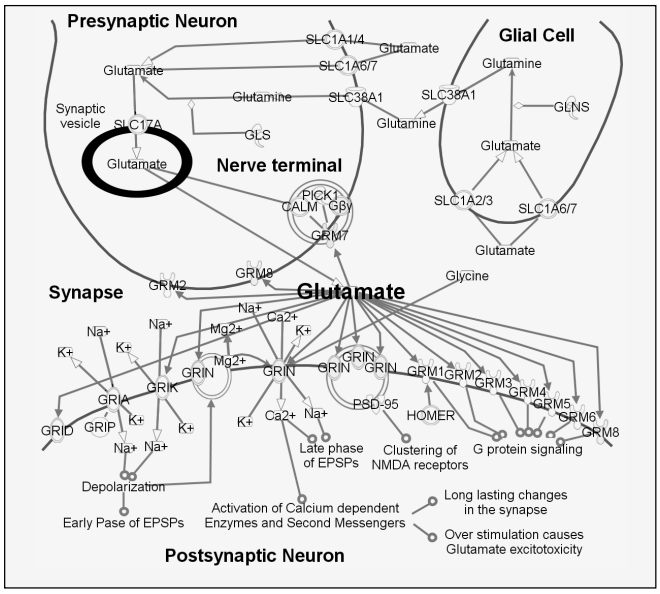
Our previous findings show that CLN treatment down-regulates mRNA levels for resistin and alters the expression genes in resistin-induced networks (19). It has been shown that resistin is engaged in inflammatory conditions in humans through IL-1, IL-6 and TNF-α (31, 32).

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 tauopathia(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.

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 wel 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.

Figure 5
CLN modulates glutamate receptor signaling

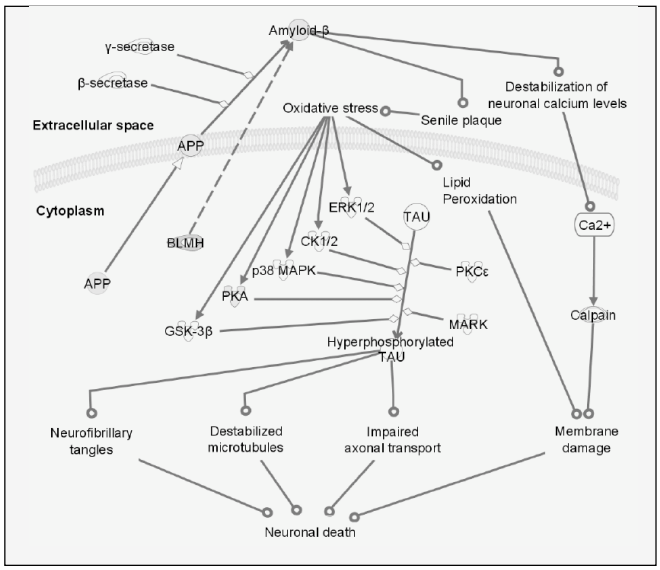


CALM:calmodulin, G beta gamma:G-protein beta-gamma subunits, GLNS:glutamine synthetas, GLS:glutaminase, GRIA:ionotropic glutamate receptor AMPA1, GRID:ionotropic glutamate receptor delta, GRIK:kainate glutamate receptor, GRIN:NMDA glutamate receptor, GRIP:glutamate receptor interacting protein 1, GRM1-8:glutamate receptor metabotropic 1-8, HOMER:homer homolog proteins, PICK1:protein interacting with PRKCA 1, PSD-95:postsynaptic density fraction protein 95, SLC:solute carrier. Network node shapes, colors, and edge types as in Fig. 1. 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).

Declining cognitive functions in Alzheimer's disease are thought to be caused by misfolded, aggregated Aβ production

and Tau hyperphosphorylation (7). 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



AKT:akt viral oncogene homolog, APP: amyloid beta precursor protein, BLMH: bleomycin hydrolase, CK1/2:casein kinase 1/2, ERK1/2:MAP kinase 1/3, GSK-3-beta:glycogen synthase kinase 3 beta, MARK:MAP/microtubule affinity-regulating kinase 1, p38 MAPK:mitogen-activated protein kinase, PKA:cAMP-dependent protein kinase, PKC-epsilon:protein kinase C epsilon, TAU:microtubule-associated protein tau. Network node shapes, colors, and edge types as in Fig. 1. 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).

Tau (a microtubule-associated phosphoprotein) hyperphosphorylation is a hallmark of AD (39, 40). 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 extra-cellularly (39, 40). Tau is hyper-phosphorylated by protein kinases, including cyclic AMP-dependent protein kinase (APKA) calcium/calmodulin-dependent protein kinase II (CK1/2), MAPK kinases (MEK1/2 and p38 MAPK), glycogen synthase kinase-3β (GSK-3β), and protein kinase Cε (PKCε) [39,40]. Down-regulation of Tau at mRNA level did not reach statistical significance after CLN treatment (Fig. 6); however, kinases implicated in its post-translational modification (phosphorylation) are significantly ($p<0.05$) affected. Our network analysis shows (Fig. 6) that CLN treatment down-regulated PKA (one of the rate-limiting kinases in Tau phosphorylation) expression. Although expressions of other kinases implicated in Tau phosphorylation were not directly affected by CLN, the expression of upstream kinases including MAPK kinases (Fig. 3b; e.g., MKK3, MKK6) in MAPK pathways were down-regulated (19).

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.

Acknowledgement: This work was supported by ReGen Therapeutics Plc, London, UK

References

1. Walton HS, Dodd PR. Glutamate-glutamine cycling in Alzheimer's disease. Neurochem Int 2007;50(7-8):1052-1066.
2. Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 2002;297(5580):353-356.
3. Kang DE, Pietrzik CU, Baum L et al. Modulation of amyloid beta-protein clearance and Alzheimer's disease susceptibility by the LDL receptor-related protein pathway. J Clin Invest 2000;106(9):1159-1166.
4. Selkoe DJ. Alzheimer's disease: genes, proteins, and therapy. Physiol Rev 2001;81(2):741-766.
5. Rogers J, Mastroeni D, Leonard B, Joyce J, Grover A. Neuroinflammation in Alzheimer's disease and Parkinson's disease: are microglia pathogenic in either disorder? Int Rev Neurobiol 2007;82:235-246.

6. Beal MF. Mitochondria take center stage in aging and neurodegeneration. Ann Neurol 2005;58(4):495-505.
7. Boldogh I, Kruzel ML. Colostrinin: an oxidative stress modulator for prevention and treatment of age-related disorders. J Alzheimers Dis 2008;13(3):303-321.
8. Bilikiewicz A, Gaus W. Colostrinin (a naturally occurring, proline-rich, polypeptide mixture) in the treatment of Alzheimer's disease. J Alzheimers Dis 2004;6(1):17-26.
9. Leszek J, Inglot AD, Janusz M, Lisowski J, Krukowska K, Georgiades JA. Colostrinin: a proline-rich polypeptide (PRP) complex isolated from ovine colostrum for treatment of Alzheimer's disease. A double-blind, placebo-controlled study. Arch Immunol Ther Exp (Warsz) 1999;47(6):377-385.
10. Leszek J, Inglot AD, Janusz M et al. Colostrinin proline-rich polypeptide complex from ovine colostrum--a long-term study of its efficacy in Alzheimer's disease. Med Sci Monit 2002;8(10):PI93-96.
11. Schuster D, Rajendran A, Hui SW, Nicotera T, Srikrishnan T, Kruzel ML. Protective effect of colostrinin on neuroblastoma cell survival is due to reduced aggregation of beta-amyloid. Neuropeptides 2005;39(4):419-426.
12. Bacsi A, Woodberry M, Kruzel ML, Boldogh I. Colostrinin delays the onset of proliferative senescence of diploid murine fibroblast cells. Neuropeptides 2007;41(2):93-101.
13. Bacsi A, Stanton GJ, Hughes TK, Kruze M, Boldogh I. Colostrinin-driven neurite outgrowth requires p53 activation in PC12 cells. Cell Mol Neurobiol 2005;25(7):1123-1139.
14. Boldogh I, Hughes, TK., Georgiades, JA., Stanton, J. Antioxidant and nerve differentiating activity of colostrinine and related peptides. In: Georgiades J, editor; 2000 December 1, 2000; Lodz, Poland.
15. Boldogh I, A. Bacsi, L. Agulera-Aguirre, P. German and M. Kruzel. Colostrinin™ Increases the Lifespan and Neurological Performance of Mice. Neurodegenerative Diseases 2007;4(Supplement):264.
16. Zimecki M, Janusz M, Staroscik K, Wieczorek Z, Lisowski J. Immunological activity of a proline-rich polypeptide from ovine colostrum. Arch Immunol Ther Exp (Warsz) 1978;26(1-6):23-29.
17. Zimecki M, Pierce A. Immunotropic properties of fractions isolated from human milk. Arch Immunol Ther Exp 1984;32(2):203-209.
18. Szaniszlo P, Wang N, Sinha M et al. Getting the right cells to the array: Gene expression microarray analysis of cell mixtures and sorted cells. Cytometry A 2004;59(2):191-202.
19. Szaniszlo P GP, Hajas G, Saenz, D., Woodberry M, Kruzel M, Boldogh I. Effects of Colostrinin on Gene Expression – Transcriptomal Network Analysis. International Immunopharmacology 2008;In Press.
20. Brigelius-Flohe R, Banning A, Kny M, Bol GF. Redox events in interleukin-1 signaling. Arch Biochem Biophys 2004;423(1):66-73.
21. Ravaglia G, Forti P, Maioli F et al. Blood inflammatory markers and risk of dementia: The Conselice Study of Brain Aging. Neurobiol Aging 2007;28(12):1810-1820.
22. Brasier AR. The NF-kappaB regulatory network. Cardiovasc Toxicol 2006;6(2):111-130.
23. Tian B, Brasier AR. Identification of a nuclear factor kappa B-dependent gene network. Recent Prog Horm Res 2003;58:95-130.
24. Kaminska B. MAPK signalling pathways as molecular targets for anti-inflammatory therapy--from molecular mechanisms to therapeutic benefits. Biochim Biophys Acta 2005;1754(1-2):253-262.
25. Schindler JF, Monahan JB, Smith WG. p38 pathway kinases as anti-inflammatory drug targets. J Dent Res 2007;86(9):800-811.
26. Saini SS, MacGlashan D. How IgE upregulates the allergic response. Curr Opin Immunol 2002;14(6):694-697.
27. Boldogh I, L. Aguilera-Aguirre, A. Bacsi, B.K. Choudhury, A. Saavedra-Molina, and M. Kruzel. Colostrinin decreases hypersensitivity and allergic responses to common allergens. International Archives of Allergy and Immunology 2008;146(1):298-306.
28. Xiang YY, Wang S, Liu M et al. A GABAergic system in airway epithelium is essential for mucus overproduction in asthma. Nat Med 2007;13(7):862-867.
29. Jin N, Narasaraju T, Kolliputi N, Chen J, Liu L. Differential expression of GABAA receptor pi subunit in cultured rat alveolar epithelial cells. Cell Tissue Res 2005;321(2):173-183.
30. Lanctot KL, Herrmann N, Mazzotta P, Khan LR, Ingber N. GABAergic function in Alzheimer's disease: evidence for dysfunction and potential as a therapeutic target for the treatment of behavioural and psychological symptoms of dementia. Can J Psychiatry 2004;49(7):439-453.
31. Guzik TJ, Mangalat D, Korbut R. Adipocytokines - novel link between inflammation and vascular function? J Physiol Pharmacol 2006;57(4):505-528.
32. Calabro P, Yeh ET. Obesity, inflammation, and vascular disease: the role of the adipose tissue as an endocrine organ. Subcell Biochem 2007;42:63-91.
33. Mehlhorn G, Hollborn M, Schliebs R. Induction of cytokines in glial cells surrounding cortical beta-amyloid plaques in transgenic Tg2576 mice with Alzheimer pathology. Int J Dev Neurosci 2000;18(4-5):423-431.
34. Cheyne JE, Montgomery JM. Plasticity-dependent changes in metabotropic glutamate receptor expression at excitatory hippocampal synapses. Mol Cell Neurosci 2008;37(3):432-439.
35. Ure J, Baudry M, Perassolo M. Metabotropic glutamate receptors and epilepsy. J Neurol Sci 2006;247(1):1-9.
36. Aizawa H, Kwak S. [ALS and excitatory amino acid]. Brain Nerve 2007;59(10):1117-1127.
37. Fan MM, Raymond LA. N-methyl-D-aspartate (NMDA) receptor function and excitotoxicity in Huntington's disease. Prog Neurobiol 2007;81(5-6):272-293.
38. Van der Schyf CJ, Gal S, Geldenhuys WJ, Youdim MB. Multifunctional neuroprotective drugs targeting monoamine oxidase inhibition, iron chelation, adenosine receptors, and cholinergic and glutamatergic action for neurodegenerative diseases. Expert Opin Investig Drugs 2006;15(8):873-886.
39. Brandt R, Leger J, Lee G. Interaction of tau with the neural plasma membrane mediated by tau's amino-terminal projection domain. J Cell Biol 1995;131(5):1327-1340.
40. Maas T, Eidenmuller J, Brandt R. Interaction of tau with the neural membrane cortex is regulated by phosphorylation at sites that are modified in paired helical filaments. J Biol Chem 2000;275(21):15733-15740.