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# COLOSTRININ: A PROLINE-RICH POLYPEPTIDE COMPLEX OF POTENTIAL THERAPEUTIC INTEREST

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**Abstract**

A proline-rich polypeptide complex (PRP) subsequently known as Colostrinin™ was found for the first time in ovine colostrum as a fraction accompanying colostral IgG2. Subsequently, similar polypeptides were found in human, bovine and caprine colostrum. PRP is a complex of peptides of molecular masses from 500 to 3000 Da. It contains 25% proline residues and 40% hydrophobic amino acids. It is not species specific, and is active both *in vivo* and *in vitro*. PRP possesses immunoregulatory properties, including effects on humoral and cellular immune responses, shows regulatory activity in Th1 and Th2 cytokine induction, and has the ability to inhibit the overproduction of reactive oxygen species and nitric oxide. PRP has also shown psychotropic properties. Both immunoregulatory and psychotropic properties suggest potential clinical use of PRP for neurodegenerative disorders. Beneficial effects of PRP/Colostrinin in the case of Alzheimer's disease were shown in double-blind placebo-controlled trials, in long-term open-label studies and in multicenter clinical trials. A very important property of PRP/Colostrinin and one of its components, a nonapeptide (NP), is the prevention of Aβ aggregation and the disruption of aggregates already formed. Moreover, PRP has been found to modulate neurite outgrowth, suppress uncontrolled activation of cells, and reduce 4-HNE-mediated cellular damage. Biological response modifying activity of PRP/Colostrinin can play an important role in its use in the treatment of Alzheimer's disease and suggests its application beyond neurodegenerative disorders.

***Key words:*** Proline-rich polypeptide complex (PRP)/Colostrinin/(CLN)™, immunomodulatory properties, cytokine induction, reactive oxygen species and nitric oxide, Alzheimer's disease.

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## INTRODUCTION

Proline-rich proteins and peptides are widespread in microorganisms, plants and animals. They can influence many biological functions such as regulation of cell and tissue processes and signal and regulatory protein interactions. The most important are the peptides with potential therapeutic significance, i.e. showing antiviral, antibacterial, antitumor, or immunoregulatory activity. Peptides with immunotropic activity, especially constituents of colostrum and milk, seem to be of great importance.

Colostrum and milk – the first mammalian nourishment – are the richest reservoir of constituents of great importance in newborn development. They contain protective and supporting factors. Immunoglobulins are a reservoir of immunity transferred from the mother to the newborn. Immunity can be transferred not only by immunoglobulins. Besides immunoglobulins, another constituent of milk, lactoferrin, has a protective function, exhibiting antibacterial, antifungal, antiviral, antiparasitic, and antitumor activities. Supporting factors such as growth factors, nucleotides, vitamins, and minerals stimulate the growth and regeneration of cartilage, nerve, bone, and muscle, and balance blood sugar. Colostrum and milk are rich in cytokines, lymphokines and peptides which may stimulate the neonate immune system and play a regulatory role.

A possibility that colostrum could be a source of immunomodulatory substances was shown for the first time in our laboratory during studies on ovine and bovine serum and colostral IgG. We have found that ovine colostral IgG2 is contaminated with a peptide with high content of proline residues (30, 41). Attempts to obtain anti-ovine IgG2 antibodies by immunization with peptide-contaminated immunoglobulins were unsuccessful. However, IgG2 separated from peptide, named proline-rich polypeptide (PRP), was a good immunogen. This indicated that PRP might have immunotropic activity. Proline-rich polypeptide, subsequently called Colostrinin™ (CLN), was also found in human, bovine, and caprine colostrum (48, 57). The yield of proline-rich polypeptide is dependent on time after delivery. The level of PRP isolated by the method of Janusz et al. (31) is the highest, between 400 and 800 mg/l, in sheep colostrum collected within the first 6 hours. PRP is also present in colostrum collected several days after delivery and in milk but at a much lower level. PRP is a complex of low-molecular-weight peptides, ranging from 500 to 3000 Da. Proline residues in PRP comprise up to 25%. It contains a high proportion of hydrophobic amino acids (40%), a low percentage of glycine, and no alanine, arginine or cysteine residues. PRP is not phosphorylated or glycosylated. Circular dichroism spectra in water and in 50% (v/v) trifluoroethanol suggest block sequences of proline residues forming helices of poly-proline II. PRP is susceptible to proteases but collagenase resistant. It is a

weak immunogen. In PRP complex peptides with homology to β-casein and its hypothetical homolog and to annexin have been found. Peptides with no significant homology to any specific protein in the current GenBank database have also been identified (31, 36). When analyzing gene expression changes it was found that Colostrinin elicited highly complex and multiphasic changes in the gene expression profile of treated cells (63). Proline-rich polypeptide complexes of both ovine and bovine origin are not species specific. They are active in mice, rats, chickens, and humans.

Two possible biological functions of PRP were considered. The first was that the polypeptide could prevent the proteolysis of colostral immunoglobulins in the digestive tract of newborns and the second that polypeptide complex could act as an immunomodulator. It was shown that the presence of PRP did not prevent proteolysis of ovine colostral IgG1 and IgG2 by trypsin, pepsin, and papain. The polypeptide did not act as an inhibitor of proteolytic enzymes with other substrates, either (31).

## IMMUNOTROPIC EFFECTS OF PRP

As shown in a mouse model, PRP complex exerted effects on the humoral and cellular immune response, both *in vivo* and *in vitro*. The effect depended strongly on the intensity of the response and not on the time of administration (67). PRP induces differentiation of cells exhibiting helper or suppressive function from glass-nonadherent and glass-adherent precursor cells, respectively. The fact that PRP may generate two opposite activities from the pool of thymocytes explains its immunoregulatory effect (42, 78, 79. It is suggested that the activity of PRP could be mediated by prostaglandins (67). Proline-rich polypeptide complex reversibly converts sensitivity of thymocytes to cortisone and activity in the GvH reaction (77). PRP acting on cortisone-sensitive (CS) or cortisone-resistant (CR) thymocytes changes the number of cells forming autologous rosettes in mice (66).

PRP is able to reduce binding of peanut agglutinin (PNA) to murine $PNA^{high}$ and to increase the binding of PNA to $PNA^{low}$ cells. The transition of binding ability can be reversed by the second treatment of cells with PRP (42). It is suggested that in the transformation of $PNA^{high}$ into $PNA^{low}$ cells β-galactosidase activated by PRP might be involved (56).

The two-way effects of PRP on PNA binding, sensitivity to hydrocortisone, and helper-suppressor activity make this polypeptide unique among the known immunomodulators. Immunomodulatory activity of PRP is observed in a dose of 1-10 μg/mouse or per $10^7$ thymocytes in a cell culture. In higher doses (>10 μg) PRP induces proliferation of T-cells from lymph nodes and splenocytes. It is suggested that in neonates polypeptide complex could replace some functions of interleukin-1 (80). The target cells for PRP are restricted to a fraction of cells not expressing CD4, CD8, or CD3 antigens, representing less than 0.5% of the total thymocyte population. These precursor cells acquire, following incubation with PRP, the cell markers present on mature T cells (68). Proline-rich polypeptide complex increases the permeability of skin vessels, which may be important for intestinal absorption in neonates (67). The involvement of suppressor T cells, generated by PRP, was also implicated in the case of an experimental autoimmune response to erythrocytes (26). PRP administered intraperitoneally into NZB mice significantly lowered the incidence of a positive Coombs reaction and prolonged the mean age of mice. From a pool of precursor cells, PRP may induce suppressor cells controlling the development of hemolytic anemia (76). Proline-rich polypeptide complex is the first protein of mammalian origin that functions as a B cell-trophic mitogen. PRP induces resting mouse B cells into and supported their progression through the cell cycle at frequencies comparable to those seen for bacterial lipopolysaccharide (35). Immunoregulatory activity similar to whole PRP complex was reflected by a nonapeptide fragment, VESYVPLFP (NP), isolated from chymotryptic digestion of PRP and obtained by chemical synthesis (32, 59).

## PROLINE-RICH POLYPEPTIDE COMPLEX INFLUENCES INNATE IMMUNITY

Innate (natural) immunity refers to the basic resistance to disease that a species possesses. It is the first line of defense against infections. The reactions of innate immunity include, among others, phagocytosis, the production and activity of cytokines, chemokines, adhesion molecules, acute phase proteins, reactive oxygen species (ROS) and reactive nitrogen species (NOS). It was shown that PRP complex did not induce the production of nitric oxide (NO) in mice; it was able to inhibit the release of NO induced by LPS by about 30-50%. This effect was accompanied by lower iNOS protein expression in peritoneal cells. In the liver sections of mice treated with PRP 6 h after LPS application, the number of iNOS-positive cells was significantly reduced (71,74). Proline-rich polypeptide complex, and at a lesser intensity its nonapeptide constituent (NP), show both an inductive and regulatory effect on cytokine and reactive oxygen species release in human peripheral blood leukocytes and whole blood cultures. In cultures of human whole blood cells, PRP in concentrations of 1-100 μg was able to induce the secretion of both Th1, i.e. IFN and TNF-α, and Th2 cytokines, i.e., IL-6 and IL-10, involved in the cellular and humoral immune response, respectively (28, 73). In human peripheral blood mononuclear cells, PRP induced IL-6, IL-10, and TNF-α and affected the release of TNF-α and IL-10 induced by LPS. PRP-derived nonapeptide (NP) induced TNF-α only, and inhibited release of IFN-γ and IL-10. PRP also inhibited the release of hydrogen peroxide ($H_2O_2$) induced by PMA and the activity of superoxide dismutase (72). Using a premonocytic HL-60 cell line which differentiates into monocyte/macrophage cells by incubation with vitamin $D_3$, it was shown that under application of PRP together with inducer, both expression of the differentiation markers CD11b and CD14 and phagocytic ability were lowered (by 30-40% and over 60%, respectively). These results suggest that PRP affecting the early stages of differentiation/maturation of cells of a monocyte/macrophage lineage may regulate the inflammatory processes in which these cells participate (37). Expression of many genes including inducible nitric oxide synthase and cytokines that affect the inflammatory process is under the control of NF-κB (43). It was shown that PRP added to human peripheral blood mononuclear cells, simultaneously or before LPS, inhibited the NF-κB activity induced by LPS. Inhibition of NF-κB activity could explain the effect on the expression of the proinflammatory cytokines and the effect on the ac-

tivity of superoxide dismutase (75).

## PROLINE-RICH POLYPEPTIDE/COLOSTRININ – PROSPECTS FOR CLINICAL USE

The immunomodulatory properties of proline-rich polypeptide complex, its effect on both acquired and innate immunity, summarized in Figure 1, point to its potential use in therapy. Its origin from a natural source – the first mammalian nourishment – suggests safety of the preparation. Indeed, in animal studies, the $LD_{50}$ for mice given Colostrinin by oral or parenteral routes was > 1.25 g/kg body weight (28), over 1000 times more than used in studies to show the effect. Moreover, in long-term studies in the senescence-accelerated mouse (SAMP1), Colostrinin administered via drinking water, in doses of 0.1 μg or 1 μg per kg per day, from 3 months of age until death, caused no adverse effects and lifespan was increased significantly in comparison to control mice (10). In preliminary experiments on NZB mice it was shown that PRP significantly lowered the incidence of positive Coombs reaction and prolonged the mean age of the mice. The effect of PRP on survival of mice was better when the treatment with PRP started when mice showed the first signs of the disease (76). Regulatory activity of PRP/Colostrinin in cytokine induction and ability to inhibit the overproduction of oxygen species and nitric oxide suggest its therapeutic use for diseases in which changes in innate immunity play a role in the pathogenesis, e.g. Alzheimer's disease.

## ALZHEIMER'S DISEASE AND THERAPEUTIC STRATEGY

Alzheimer's disease (AD) is one of the most widespread neurodegenerative disorders causing cognitive impairment in older persons. Chronically activated microglia and astrocytes can release highly toxic products such as reactive oxygen intermediates, nitric oxide, inflammatory cytokines, proteolytic enzymes, complement factors, and excitatory amino acids (24, 52). Cytokines such as TNF-α, IL-1β, and IL-6, when chronically produced at high concentrations, can be directly cytotoxic or can also stimulate the synthesis of βAPP and Aβ peptides, thereby favoring the formation of Aβ fibrils (16, 44, 60). TNF-α and IL-β can play a dual role in producing either neurodegeneration or neuroprotection in the central nervous system (47, 55). Multiple lines of evidence demonstrate that increased brain oxidative stress is a key feature of AD and is a marker of neuronal degeneration. Nitric oxide in the brain is involved in normal physiological functions, but its overproduction in pathological processes leads to tissue damage (13, 14, 21, 29, 51). Moderate to severe Alzheimer's disease is characterized by increasing cognitive, functional and behavioral dysfunction.

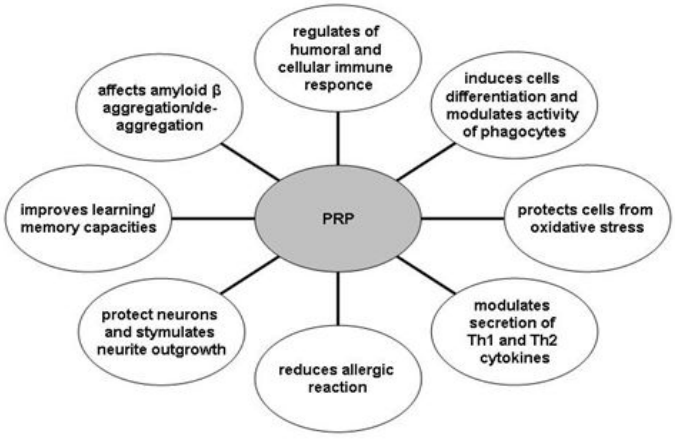


**Figure 1.** Effects of proline-rich polypeptide complex (PRP).

In spite of significant progress in understanding the pathophysiology of AD, there is still no fully effective therapeutic strategy. In AD patients the progressive reduction of levels of neurotransmitters, in particular acetylcholine, is reduced in specific regions of the brain. Thus, at present the standard care of patients involves the reversible acetylcholinesterase inhibitors donepezil (Arizept), rivastigmine (Exelon), and galantamine (Reminyl). The application of both rivastigmine and donepezil is stopped if adverse effects and limited efficacy are observed in patients after four-year treatment (19, 54). To avoid some adverse effects, the use of rivastigmine in the form of transdermal patches was proposed (69). It may also be used in combination with cholinesterase inhibitors (18, 27). Some antioxidants, such as flavonoids and vitamins and nonsteroidal anti-inflammatory drugs (NSAIDs), appeared to delay or slow the clinical expression of AD (1, 46). Other therapeutic approaches are to use secretase inhibitors (65) or ion channel blockers (2), and prevent or reverse Aβ misfolding and aggregation (17). Therapeutic strategies have also included the inhibition of tau hyperphosphorylation (23), neuronal and stem cell transplantation (20, 38) and immunotherapy (58, 64, 70). Compounds of natural origin, such as extracts from *Ginkgo biloba* and *Salvia miltiorrhiza*, as well as polyphenolic components of red wine, could be used for the prevention and/or therapy of AD (3, 25, 45). Drugs that can cross the blood-brain barrier including antisense nucleotides, regulatory proteins, and peptides are among the greatest challenges in medicine today (4, 34).

## POTENTIAL MECHANISM OF PRP/COLOSTRININ ACTION IN ALZHEIMER'S DISEASE

The presented results indicate that proline-rich polypeptide complex provides a combination of immunoregulatory and antioxidative effects. It shows multidirectional activity, concomitantly affecting the immune and nervous system.

It was of interest to check if Colostrinin can affect learning, memory and lifespan. The effects on spatial and incidental memory were studied in rats. It was shown that Colostrinin at low doses (4 μg per animal) facilitates the acquisition of spatial learning of aged, 13-month-old, but not young, 3-month-old animals. Analysis of swimming behavior of animals subjected to the Morris Water Maze test indicated that the aged rats were impaired in the latter stages of spatial learning. Treatment of rats with Colostrinin resulted in significantly higher precision in the animals' ability to find the platform, as revealed by the higher number of direct hits (49). In similar studies using one of Colostrinin's components, NP, it was shown that in aged rats which demonstrate a learning deficit, the nonapeptide may delay the extinction of long-term memories (50). It was also shown that Colostrinin administration to mice improves age-associated locomotion, and learning/ memory capacities (10). Colostrinin, independently of the route of injection, showed a cognitive-enhancing effect in

studies on newly hatched chicks (61).

In the pathogenesis of Alzheimer's disease (AD), overproduction and/or decreased degradation of Aβ leads to the oligomerization of Aβ in protofibrils. Emerging oligomers form extracellular amyloid deposits. One of the therapeutic strategies could be prevention or modulation of Aβ aggregation. It was shown that PRP complex acts as a β-sheet breaker and thus inhibits the aggregation of amyloid β peptides. Observations of Aβ (1-40) cluster formation in optical and electron microscopy reveal that in a short time of incubation (48 h), the formation of short Aβ fibrils was inhibited in the presence of Colostrinin. Upon long-term incubation with Colostrinin, amyloid fibrils were largely dissolved (53). A reduction of Aβ40 fibril formation by Colostrinin in a dose- and time-dependent fashion was also observed using Thioflavin T (12). In atomic force microscopy (AFM) and circular dichroism (CD) studies it was found that one of the PRP complex constituent peptides – the nonapeptide VESYVPLFP – can directly interact with amyloid β (Aβ42). Therefore it can not only inhibit aggregation of Aβ and formation of fibrillar structures but also can dissolve aggregates already formed, acting as a β-sheet breaker (33). This property may be of general significance. It can be assumed that the beneficial therapeutic effect of Colostrinin is connected with prevention of formation of various amyloid-like β-sheet containing aggregates *in vivo* and dissolving complexes already formed. The toxicity induced by aggregated forms of Aβ was reduced in the presence of Colostrinin and NP (33, 53).

## COLOSTRININ – CLINICAL TRIALS

The implications of therapeutic strategies in the case of Alzheimer's disease were dramatically affected by viewing AD as a multifactorial disease state. According to this view, one specific treatment may not be effective in preventing or reversing the disease status. Better efficacy could be obtained by multidrug therapy. It seems obvious that a multifunctional drug could be used as a very advantageous strategy. The properties of PRP/Colostrinin, its role in the development of the immune system and cognitive function, indicated its potential use in the treatment of neurodegenerative disorders. Therefore, the PRP complex was proposed for the treatment of Alzheimer's disease (28, 39). A double-blind placebo controlled trial was conducted to evaluate safety and efficacy. In this first clinical study 46 patients with AD were divided into 3 groups and randomly assigned to receive orally either Colostrinin (100 μg per tablet, every second day), commercially available bioorganic selenium (100 μg per tablet, every second day) or placebo tablets. One cycle of treatment lasted 3 weeks and was separated from the next cycle by a 2-week hiatus (3+2). Each patient received 10 cycles of treatment during the year of the clinical trial. The PRP-treated patients, in contrast to those receiving placebo, showed an improvement in or at least stabilization of health status. The efficacy of the PRP/Colostrinin treatment depended on the stage of development of the disease. Patients who at the beginning of the trial were at an early stage of the disease responded to the Colostrinin treatment better than those with very advanced disease. Adverse reactions, if any, were remarkably mild. They included anxiety, logorrhea, and insomnia, and they subsided spontaneously within a period of 3-4 days (39). The efficacy and safety of the PRP complex was ascertained during a long-term (16- to 28-month) open-label study (40). The largest study to confirm the potential value of PRP complex in the treatment of AD was undertaken on 105 patients from six psychiatric centers in Poland. The trial consisted of a 15-week double-blind phase comparing Colostrinin with placebo, followed by a second 15-week open-label phase when all patients received Colostrinin. All patients were outpatients. They received the same dose of 100 μg and according to the same scheme (3+2) as in preliminary studies. This cycle was repeated three times for each phase. The primary efficacy outcome was assessed by the Alzheimer's Disease Assessment Scale-cognitive portion (ADAS-cog) and Clinical Global Improvement (CGI). The outcome measures included Instrumental Activities of Daily Living (IADL), Mini-Mental State Examination (MMSE), ADAS-non cognitive test and Gradation of Overall Patient Response. The full sample analysis (FSA) showed a stabilizing effect of Colostrinin on cognitive function (ADAS-cog, $p=0.02$) and on daily function (IADL, $p=0.02$). Patients graded as mild on entry showed a superior response of ADAS-cog compared with more advanced cases ($p=0.01$). The overall patients' response analysis indicates that Colostrinin has potential value in the treatment of AD, especially when given to patients in the early stages of the disease (8). It is noteworthy that any adverse reactions were remarkably mild; they included anxiety, logorrhea, and insomnia, and they subsided spontaneously within the short period of 3-4 days.

After analysis of the pathogenic phenomena in the case of AD (Figure 2) and the properties of PRP/Colostrinin known so far, we can assume that the beneficial clinical effects besides the direct effect on Aβ aggregation could also be connected with the modification of cytokines, reactive oxygen species release (72, 73) as well as the functional/phenotypic differentiation of cells (37). Moreover, it was shown that pre-treatment of human neuronal SHSY-5Y cells with Colostrinin confers neuroprotection against Aβ-induced neurotoxicity (15). Colostrinin also relieves Aβ-induced cytotoxicity in rat primary hippocampal neuronal cells and affects neurite outgrowth (6, 22).

## FUTURE DIRECTIONS FOR PRP/COLOSTRININ THERAPEUTIC USE

The mode of PRP/Colostrinin action is not fully understood because of its heterogeneous structure. However, a substantial body of evidence can shed light on mechanism of its action. In a model of cultured PC12 cells it was shown that treatment with polypeptide complex reduced the levels of 4-HNE-protein adducts, reduced intracellular levels of ROS, inhibited 4HNE-mediated GSH depletion and inhibited the activation of c-Jun N-terminal kinase (JNK), which are central to cell proliferation, differentiation and apoptosis. PRP can play a role in the regulation of cellular redox status, glutathione metabolism, and modulation of reactive oxygen species-induced signaling-mediated downstream events, for example JNK and p53 (33). Upon treatment with Colostrinin, cells ceased to proliferate and extend neuritis (6, 11). Using murine diploid fibroblast cells as an *in vitro* model for cellular aging it was found that Colostrinin significantly decelerates the senescence of cultured cells and increases their population doubling levels. It is associated with a decrease in the in-

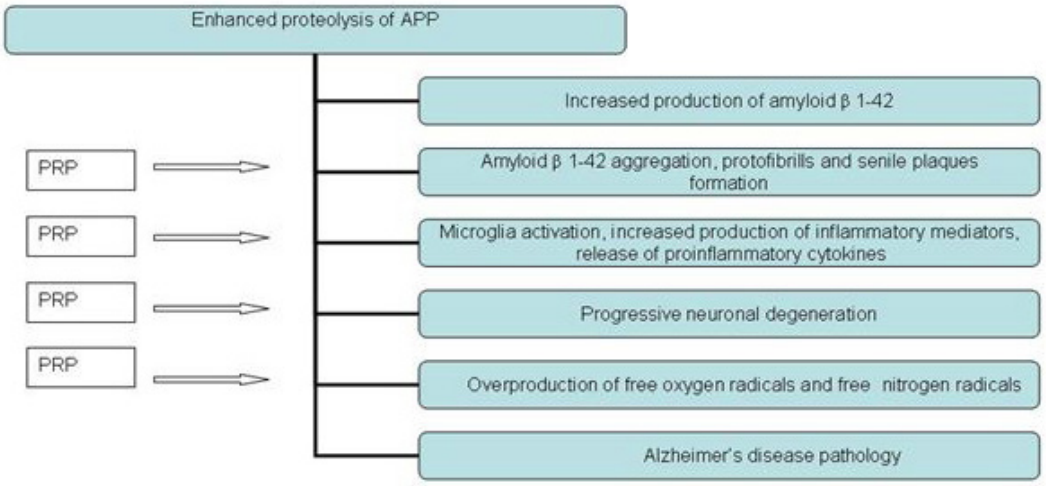


**Figure 2.** Pathogenic factors in Alzheimer's disease and therapeutic effects of PRP/Colostrinin™

tracellular level of reactive oxygen species (7). Colostrinin itself has no mutagenic activity, but it significantly lowers the mutation frequency that develops spontaneously or is induced by ROS, and chemical and physical agents (5). It was shown that Colostrinin did not increase IgE/IgG1 levels or induce a cutaneous hypersensitivity reaction, airway inflammation or mucin production. In the presence of Colostrinin IgE/IgG1 production was significantly decreased as well as airway eosinophilia, mucin production and hypersensitivity induced by allergenic extracts from ragweed pollen and house dust mites (9). The expression of many genes, including those for inducible nitric oxide synthase and cytokines that affect the inflammatory processes, are under the control of the transcription factor NF-κB. It was found that in the presence of PRP the relative level of NF-κB was lowered (75). The inhibitory effect of PRP on NF-κB activity might, at least in part, contribute to the beneficial effects.

Properties of PRP complex/Colostrinin (Fig.1) suggest its broader, beyond neurodegenerative disease, therapeutic use. The possible mechanisms by which PRP/Colostrinin exerts its biological effects could be explained by a modulatory effect in the gene expression profile. Most of the genes follow a biphasic expression pattern and the overall gene expression pattern is the sum of the sequentially up- and down-regulated gene cluster. Colostrinin mediated changes in gene expression are related to:

- immune system development and function (regulation of inflammatory pathways, generation of inflammatory cytokines and chemokines in response to antigenic/allergic stimuli, prevention of the development of airway inflammation, hyperresponsiveness and mucin production);
- nervous system function and associated diseases (effects on the canonical pathway of Aβ processing, effect on ROS generation, downregulation of key kinases);
- stress-related pathways (effect on antioxidant enzymes, regulation of glutathione metabolism, downregulation of caspase family, effect on MAPK pathway, downregulation of TGF-β and IL-1β);
- effect on resistin-leptin networks.

It is suggested that some proline-rich polypeptides, constituents of Colostrinin, bind to the cell membrane or intracellular receptors and activate cascades of signaling leading to changes in expression of gene networks (62,63).

## CONCLUSIONS

It seems clear that a multifunctional drug such as the proline-rich polypeptide complex Colostrinin could be used as a very advantageous strategy. Colostrinin, a component present in the colostrum and milk, could be not only important for the development of the immune and nervous system in newborns; it could also be useful in improving the health status of elderly persons with neurodegenerative impairment. PRP/Colostrinin-induced multiphasic changes in the gene expression profile suggest its wider application, including non-neurodegenerative disorders such as inflammatory and cardiovascular disorders, diabetes and obesity.

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