NEW TARGETS FOR PUTATIVE NEUROPROTECTIVE AGENTS

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Abstract: A better understanding of intracellular pathways engaged in the neuronal cell death afforded us new targets for developing putative neuroprotectants. Also pharmacological or genomic intervention aimed to modulate the expression of endogenous neuroprotective or toxic agents is a very promising strategy. Taking into account enormous complexity of biochemical cascades involved in neurodegenerative processes, multipotential or combined pharmacological approaches seem to be more efficient in combating degenerative brain diseases. Moreover, an improved cell transplantation also adds to a plethora of methods, which are used to afford neuroprotection and promote neurorestoration. All those strategies are reviewed in the presented article.

Besides acute brain injuries, of which stroke is the most common and harmful for normal brain functioning, there are several forms of slowly progressive brain disorders, which cause degeneration of specific population of neurons. In Amyotrophic Lateral Sclerosis (ALS), Alzheimer’s (AD), Parkinson’s (PD) and Huntington’s (HD) diseases, cholinergic motor neurons, pyramidal cortical and hippocampal neurons, nigrostriatal dopaminergic and striatal GABA(γ-aminobutyric acid)-containing neurons are affected first, respectively. Incidence of neurodegenerative diseases in human population (excluding their genetic forms) is increasing with ageing. Similar disturbances affect normal function of all neurons but only the most vulnerable of them are lost. Why some population of neuronal cells are more prone to die, it is still not fully explained. Some hypotheses point to excitotoxicity and dysregulation in calcium homeostasis, and consequent perturbations in mitochondrial function, activation of proteolytic enzymes, increased production of deleterious reactive oxygen species (ROS) and impaired cell defense system, especially in large neurons with long axons in certain brain regions. Cytoskeletal disruptions, protein aggregations, deficiency of neurotrophic factors and accompanying inflammation, are observed in all kinds of neurodegenerative disorders are results and equally triggers of vicious circle of neuronal cell death (1). Moreover, the presence of free iron and other transition metals could also facilitate neurodegeneration in specific brain areas (e.g. substantia nigra in PD) by increasing production of reactive oxygen species (2). Therefore, there have been two different strategies for preventative and therapeutic interventions in neurodegenerative disorders. One strategy is to block the disease-specific events that are believed to initiate the neurodegenerative process (e.g. anti-amyloid strategy in AD or increasing dopamine level in PD) (3-5), however, these new symptomatic therapies still do not address the fundamental problem underlying neurodegenerative diseases, the continual loss of specific populations of neurons. Alternatively, neuroprotective strategies aim to halt or slow the progressive degenerative processes. A plethora of different agents have been studied in cellular and animal models of neurodegenerative diseases, but only few of them reached clinical trials. Putative neuroprotectants are searched among substances which could delay or stop degeneration of neurons at a particular stage of neuronal cell death by inhibiting for example excitotoxicity, apoptosis, ROS production, mitochondrial perturbations or inflammation. Not fully understood mechanism of neuronal cell death (6), heterogeneity of animal models of neurodegenerative diseases, which are often inadequate to represent a human disease and inappropriate design of clinical trials, are the main reasons of the lack of efficiency of a preclinically studied neuroprotective substances in human treatment (7-9). In spite of big effort devoted to explaining biochemical and molecular changes during neuronal cell death and to constructing better animal models of human brain disorders, there is a constant search for substances with multidirectional action on several steps of neuronal cell death or for a combined treatment with few neuroprotective drugs (10, 11). Besides a pharmacological intervention, genomic approaches are intensively studied that aim to delay or stop neuronal cell death or to strengthen cellular defense system, e.g. antisense strategy, gene silencing methods, overexpression of endogenous neuroprotective proteins. Restorative therapy is another way to combat neurodegenerative diseases and could involve cell therapy or methods which increase neurogenesis, and as a result, normal neuronal activity could be restored in the damaged brain regions. These new neuroprotective pharmacological approaches as well as genomic and cellular restorative methods are the subject of the article presented herein.

Double-faced neuronal cell death and implications for neuroprotection

Acute brain injuries and stroke are accompanied by a rapid, necrosis-like cell death in the damaged region and slowly progressing, apoptosis-like neuronal degeneration in the neighboring area (12). Neuronal loss in age-related, chronic neurodegenerative diseases is also evoked by induction of the apoptotic pathway by several endogenous or exogenous factors. In some cases, it is difficult to unequivocally define the type of cell death, and a continuum of necro-apoptotic processes is observed, which has implications to a proper treatment strategy. Furthermore, the inhibition of one form of cell death (necrosis) could accelerate another (apop-
disorders with dysfunctional mitochondria (17-19). The formation of free radicals is frequently associated with ischemia, reperfusion and intracranial hemorrhage. The level of pro-apoptotic proteins was elevated, which could be a target for searching new anti-apoptotic substances. The level of pro-apoptotic (Bax, Bad, Bid) and anti-apoptotic (Bcl-2, Bcl-xl) proteins from the Bcl-2 protein family, and the presence of endogenous inhibitors of these enzymes or directly influence chromatin structure, giving characteristic morphological changes, such as the apoptotic nuclear fragmentation, cellular blebbing and formation of apoptotic bodies (6). Recently, it has been shown that the released cytochrome c could interact with endoplasmic reticulum receptors (IP3R) and in this way can accelerate calcium-dependent apoptosis (15), with endoplasmic reticulum receptors (IP3R) and in this way can accelerate calcium-dependent apoptosis (15), which suggests the existence of complex interactions between proteins in the cell death pathways and could be a target for searching new anti-apoptotic substances.

The level of pro-apoptotic (Bax, Bad, Bid) and anti-apoptotic (Bcl-2, Bcl-xl) proteins from the Bcl-2 protein family, and the presence of endogenous inhibitors of apoptosis (XIAPs, C-IAPs) decide whether neuronal cell would die by apoptosis or not (1, 16). In different kinds of neurodegenerative disorders, the level of anti-apoptotic proteins was observed to be decreased, anti-apoptotic cell defense system was impaired while the level of pro-apoptotic proteins was elevated, which facilitated the apoptotic neuronal cell death upon harmful stimuli. Moreover, inflammatory processes, which are a source of deleterious ROS, nitric oxide and cytokines, also accompanies degenerative brain disorders. The formation of free radicals is frequently associated with ischemia, reperfusion and intracranial hemorrhages and also accompanies chronic neurodegenerative disorders with dysfunctional mitochondria (17-19). The risk of free radical-induced damage is very high in the brain, which is due to its high oxygen demand, high level of iron and unsaturated fatty acids, which are substrates for production of reactive lipid radicals (20).

Neuroprotective strategies

Anti-excitotoxic approaches. So far, classical anti-excitotoxic pharmacological intervention in rapid cell death mechanisms was obtained by the inhibition of Ca²⁺ influx mainly through NMDA receptors. Although, different kinds of NMDA receptor antagonists have shown neuroprotective action under experimental conditions, they were useless in clinical practice because of their negative influence on normal brain function (competitive and noncompetitive antagonists of NMDA receptor impair memory and learning processes) or strong adverse effects (NMDA channel blockers elicit psychomimetic and amnesic action) (21, 22). Among NMDA antagonists, only memantine, a low-affinity voltage- and use-dependent open channel blocker, is widely used in the treatment of moderate to severe AD with combination with acetylcholinesterase inhibitors, but still there is no clear evidence of its neuroprotective action in AD patients (only symptomatic improvement).

Potency of memantine as neuroprotective drug is currently also studied in clinical trials for stroke, glaucoma, vascular and HIV-related dementias and second-generation memantine derivatives, which will have better neuroprotective activity have been proposed (e.g. nitromemantine, a combination of memantine with nitroglycerin, which also down regulates NMDA receptor activity) (21). Moreover, it was shown that memantine prolonged survival in the amyotrophic lateral sclerosis mouse [SOD1(G93A)] model (23). An abundant number of modulatory sites on NMDA receptor and its specific subunit composition could also be a target for drug treatment, and new specific agents with such profile are searched for (e.g. polyamine and redox site modulators, NR2B antagonists, glycine B receptors modulators) (24-26). Furthermore, AMPA receptors have regained recently attention, since their new allosteric modulators were proposed, and some new data on the mechanism of AMPA receptor activation, which could contribute to neuroprotection, were reported (27-29). AMPA receptor agonists are tested in AD, as agents which improve learning and memory (30). Furthermore, an AMPA receptor antagonist, talampanel (LY-300164) has been reported to have beneficial effect in non-human primate models of Parkinson’s disease and in ischemic rats (10, 31). Modulation of AMPA receptor could also be achieved by influencing proteins anchoring AMPA receptor in synapses (PSD-95, GRIP, PICK, NSF) and is also preclinically investigated (31). Experimental studies concern also metabotropic glutamate receptors (mGluR) which modulate activity of ionotropic glutamate receptors (group I mGluR) and glutamate release (group II and III mGluR), which could contribute to neuroprotection (32-35). To date, only one agonist of mGluR4, L-AP4 has been tested in clinical trials for PD and development of potent, selective allosteric modulation...
tors with appropriate pharmacokinetic properties is under way (36). Acute brain injuries could also be experimentally attenuated by blocking the voltage dependent calcium and sodium channels or by using potassium channel activators. Riluzole, the only approved drug for ALS, acting as an anti-glutamatergic agent by blocking voltage-dependent sodium channels delays progress of the motor neuron degeneration (37). Another – Na+ blocker and K+ activator (lubelozole and BMS-204352) were studied in clinical trials for stroke, but they showed no efficacy despite promising pre-clinical studies (7). Recently, it has been reported that during ischemia, the calcium permeable acid-sensing ion channels (ASICs) are activated, and blocking these receptors could be a new potential therapeutic target for alleviation of stroke-induced damage (38, 39).

**Antioxidants and mitochondrial stabilizers.** The best known neuroprotectants and free radical-neutralizing agents include polyphenols, e.g. quercetin, vitamin E, lazaroids and endogenously produced melatonin and estrogens (53, 54). Since melatonin, a hormone secreted from the pineal gland has been reported to have antioxidant and metal–chelating properties, its dietary supplementation seems to be beneficial to slowing neurodegenerative changes associated with the brain aging (55). A combination of iron chelation and antioxidant therapy may be one of significant approaches to neuroprotection. Multifarious neuroprotective activities of green tea catechins, have brain-permeable, nontoxic, transition metal (iron and copper)-chelatable/radical scavenger properties, that were shown in a wide array of cellular and animal models of neurological diseases (56). New generation of synthetic antioxidants, such as tirilazad, ebseled, edarawon and NXY-059 have also additional metal-chelating properties, and they have been intensively studied in both animal models of ischemia and in clinical trials. Only edarawon has been accepted for stroke treatment in Japan, whereas NXY-059 is now in phase III clinical trials (57). It should be mentioned here that some antioxidants such as vitamin C may in the presence of iron via Fenton’s reaction increase hydroxyl radical formation and promote oxidative lipid damage (48). With respect to other kinases having a potential of neuroprotection, one should mention the AMP–activated kinase (AMPK), which is the main sensor of intracellular energetic balance and is activated and phosphorylated during hypoxia. An inhibitor of AMPK, C75 has decreased ischemic brain injury, whereas an activator of this enzyme showed opposite effect (49). Also various cell-permeable calpain inhibitors have been synthesized for pharmacological inhibition of calpain activity and some of them have shown significant neuroprotection in animal models of the CNS injuries and diseases, indicating their therapeutic potential (50, 51). Since alterations in the expression of Bcl-2 family members occur in several neurodegenerative diseases (AD, PD, ALS, HD, TBI, stroke), methods to increase the overall expression and/or function of anti-apoptotic Bcl-2 family members, and thus promote neuronal survival, are extensively studied. Most treatment efforts focus either on the targeted delivery of anti-apoptotic Bcl-2 family members into the affected brain regions of interest via viral vectors, the generation of direct interactions of low molecular weight inhibitors with pro-apoptotic Bcl-2 family members, or the induced expression of Bcl-2 family members secondary to pharmacological manipulation (safe and efficacious Bcl-2 family mimetics) (52).

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toxins, beta-amyloid or ischemia by improving ATP production (61).

Anti-inflammatory strategies. Considering the involvement of inflammatory processes in mechanism of neurodegeneration, one should keep in mind that activated microglia and astrocytes are a rich source of oxygen radicals, nitric oxide and neurotoxic and proinflammatory cytokines. Nitric oxide has both neurotoxic and neuroprotective properties. It may attenuate neuronal damage through inhibition of NMDA receptor activity. On the other hand, nitric oxide can be transformed to peroxynitrite, which inactivates numerous proteins including glutamate transporter, destroys DNA and deenergizes mitochondria, ultimately leading to necrotic and apoptotic cell death (20). Clinical trials with inhibitors of nitric oxide synthase in patients with stroke have not been successful, whereas some nitric oxide donors and its precursor L-arginine increase perfusion and decrease ischemic brain damage. Although the above data give rationale for further trials with nitric oxide donors, there are some doubts about their clinical utility, since these substances possess a rather narrow therapeutic time window (62, 63). Cyclooxygenases, COX-1 and COX-2 mediate the first step of prostaglandin synthesis from arachidonic acid. Some data suggest that inducible COX-2 and the main product of its enzymatic activity, prostaglandin E2, participate in ischemia-related neuronal damage. In vitro studies showed that the stimulation of EP2 prostaglandin receptors, in cAMP-dependent manner protected neuronal cells against damage evoked by NMDA or oxygen/glucose deprivation. These encouraging data have been confirmed in an animal model of transient brain ischemia, which is in agreement with high level of EP2 receptor expression in the neocortex, hippocampus and striatum (64). Several epidemiological studies have indicated that the long-term use of NSAIDs, most of which are cyclooxygenase (COX) inhibitors, may reduce the risk of Alzheimer’s disease. For this reason, anti-inflammatory COX-inhibiting NSAIDs have received increased attention in experimental and therapeutic trials for Alzheimer’s disease. However, several recent efforts attempting to demonstrate a therapeutic effect of NSAIDs in Alzheimer’s disease have largely failed (65). Immunophilin ligands, such as FK506 and cyclosporin A, used in immunosuppression, were found to be neuroprotective in several models of neuronal cell injury (12). FK506 has been shown to interfere with the apoptotic pathway of neuronal cells, since it inhibited JNK activity, cytochrome c release, caspase-3 activation, and CD95 ligand expression. Those observations were a clue to synthesis of new immunophilin ligands, such as GPI-1046 and V-10,367, which are immunosuppressive derivatives of FK506. They have been shown to exert neuroprotective and neuroregenerative actions in several systems and could serve as therapies for neurodegenerative disorders (66, 67). Inflammatory mediators, such as TNFalpha and FasL play an important role in apoptosis in ALS and their expression is elevated in lumbar spinal cord section of both ALS patients and transgenic animal model of this disease. It has been found that thalidomide and lenalidomide prolong survival in the transgenic mouse model of ALS (G93A mice) by destabilizing DNA coding for TNFalpha and other cytokines (68), thus being a potential drug for treatment ALS (69). Interestingly, the same group of investigators reported that also pioglitazone, a peroxisome proliferator-activated receptor-gamma agonist with anti-inflammatory properties, improved motor performance, delayed weight loss, attenuated motor neuron loss, and extended survival of G93A mice. Pioglitazone appears to have therapeutic potential for human ALS, since it also reduces iNOS, NFkB, and 3-nitrotyrosine immunoreactivity in the spinal cords of G93A transgenic mice (70). Some antibiotics could also be beneficial in some brain diseases, for example, minocycline, which possesses anti-apoptotic and p38 MAPK inhibitory activity, was neuroprotective in acute brain injuries and ALS animal models and is currently studied in clinical trials (71-74). Given the pathogenic impact of oxidative stress and neuroinflammation, therapeutic strategies aimed to blunt these processes are considered an effective way to confer neuroprotection. Recently, the nuclear transcription factor Nrf2, that binds to the antioxidant response element (ARE) in gene promoters, has been reported to constitute a key regulatory factor in the co-ordinate induction of a battery of endogenous cytoprotective genes, including those coding for both antioxidant- and anti-inflammatory proteins suggesting that targeting the Nrf2/ARE pathway may represent a novel therapeutic approach to the development of a new class of neuroprotective drugs (75).

Multipotential agents and combined pharmacotherapy. It appears that the successful treatment of neurodegenerative diseases can be achieved by combining a few drugs, which have neuroprotective properties. Such combinations could include for example antagonists of glutamate receptors with inhibitors of apoptosis, or with antioxidants or anti-inflammatory agents (7, 10, 21). Since dysfunction in acetylation homeostasis could evoke neurodegeneration, agents which restore balanced acetylation status could diminish cell death (76). For example, the histone deacetylase inhibitor phenylbutyrate (PBA) was effective in the G93A transgenic mouse model and a combination of this drug with the catalytic antioxidant AEO1 10150 accelerated neuroprotection (77). In the same ALS model, the combination of creatine with COX-2 inhibitors produced additive neuroprotective effects and extended survival (78). Moreover, new, anti-apoptotic properties of drugs clinically used in symptomatic treatment of neurodegenerative diseases (memantine, selegiline, antiplatelet drugs) are being discovered and are used in constructing new multipotential drugs (11, 21, 79) For example ladostigil, a derivate of rasagline (monoaminooxidase B inhibitor) with acetylcholinesterase inhibitory activity is tested in AD and PD (11). In addition, a multipotential drug, such as dexanabinol (synthetic cannabinoid with NMDA antagonist, antioxidant and anti-TNF properties) and AM36 (Na+ blocker, NMDA receptor antagonist and anti-apoptotic agent) are tested in stroke (7). Other drugs, statins (HMG-CoA reductase inhibitors], exert cholesterol-independent pleiotropic effects that include anti-thrombotic, anti-inflammatory, and antioxidant properties, and specific anti-excitotoxic effects independent of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibition, which has potential therapeutic implications (80). Beside exoge-
ous substances with the multipotential neuroprotective properties in vivo and in vitro. They protect neurons against different insults, such as anoxia, oxidative stress, hydrogen peroxide, iron or amyloid-β peptide (81), however, their effects seem to depend on the type of neuronal tissue, stage of its development and neurotoxic factors. Although estrogens have been found to be especially important regulators of mitochondrial apoptotic pathway (81-84), they appear also to be involved in cytokine-mediated apoptosis. Recent data suggest that estrogens interfere with cytokine effects on the central nervous system (CNS) by affecting cytokine signaling components, such as nitric oxide (NO) synthesis and activation of transcription factor NFκB (85-87). Astrocytes, which play a key role in estrogen-induced synapse formation, plasticity, and neuronal morphology, are the main target of estrogen in the brain (88, 89). It is possible that by releasing neuroprotective factors, such as transforming growth factor (TGF-β1, TGF-β2) or glial cell-derived neurotrophic factor (GDNF), astrocytes, potentiate neuroprotection attributed to estrogen (90, 91). Under physiological conditions, IL-1β may have a neuroprotective action through positive modulation of nerve growth factor (NGF) (92). Since the hippocampus is more abundant in IL-1 receptors than the neocortex, it could also be more effectively protected by estradiol, which is known to interact with growth factors, such as brain-derived neurotrophic factor (BDNF), insulin-like growth factor-I (IGF-I) or NGF and their receptors (93). An inflammatory reaction that contributes to the tissue damage after cerebral ischemia or to the progression of neurodegenerative disease is mediated in part by toxic amounts of nitric oxide (NO) and exaggerated production of pro-inflammatory cytokines. Estrogens were found to inhibit inflammatory reaction in astroglial and microglial cells, possibly via estrogen receptor-dependent activation of MAP kinase (94). Estrogens are neuroprotective in a variety of in vitro and in vivo models of cerebral ischemia (95). There is a body of evidence indicating that during brain ischemia the physiological estrogen stimulation results in an increased release of vasocondensing substances, which improve brain metabolism and cerebral blood flow. Estrogen-mediated neuroprotection against ischemia likely involves the activation of gamma PKC through the G-protein-coupled estrogen receptors on the plasma membrane (96). However, the non-estrogen receptor mechanism has also been suggested (97). There is a number of evidences suggesting a direct interference of estrogens with apoptotic processes, especially those triggered by mitochondrial pathway. Estradiol has been found to stimulate the expression of anti-apoptotic proteins, like Bcl-2 or Bcl-xl, which prevent cytochrome c efflux from mitochondria to cytoplasm that, finally, results in the inhibition of caspase-dependent apoptotic cell death. Estrogen-induced Bcl-2 up-regulation was found in arcuate nucleus neurons of female rats in vivo (82) and in NT2 neuronal cells in vitro (98), while estradiol effects on Bcl-xl were observed in vivo and in vitro in hippocampal and cortical cells (99). An anti-apoptotic action of estrogens may also depend on suppression of pro-apoptotic gene transcription, such as Nip-2 or Bad (100-103), possibly through the AP-1 site downstream of c-Jun amino N-terminal kinases (JNKs) and caspase-3 activation, as indicated in nigral dopaminergic neurons in rat primary cultures (104). Recently, estradiol has been found to prevent caspase-6-mediated neuronal cell death, possibly by inducing a caspase inhibitory factor (CIF) through a receptor-mediated, but non-genomic, pathway (105). Moreover, estrogens can counteract apoptotic insults by affecting the basal levels of tau phosphorylation at a site known to be phosphorylated by glycogen synthase kinase-3β (GSK-3β), thereby acting as antioxidants and interacting directly with cellular and mitochondrial membranes (106-109).

Finding the most suitable and effective protective strategy needs evaluation of risk-to-benefit ratio. Thus, issues of thrombotic and neoplastic risks must be factored into the design of estrogen alternatives for hormone replacement therapy. Selective estrogen receptor modulators (SERMs) may represent an alternative to estrogen for the treatment or the prevention of neurodegenerative disorders. SERMs represent a class comprising a growing number of compounds that act either as estrogen receptor agonists or antagonists in a tissue-specific manner distinct from that of estradiol. Neuroprotection attributed to estradiol is associated with a strong down-regulation of reactive astroglia, while SERMs do not affect reactive gliosis (110). SERMs presently in use are tamoxifen and raloxifene. Next-generation SERMs taken into clinical studies include idoxifene, droloxifene, ospemifene, arzoxifene, acolbifene/EM-800, levomeloxifene, lasofoxifene (CP-336,156), bazedoxifene (TSE-424) and HMR 3339 (111).

Multiple issues regarding the timing, formulation and duration of the estrogen therapy intervention remain unresolved. The results from hormone replacement studies in humans are thus far inconclusive. A possible alternative to hormonal replacement therapy is to increase local steroidogenesis by neural tissues, which express enzymes for steroid synthesis and metabolism. The rate-limiting step in steroid biosynthesis is the intramitochondrial transport of cholesterol, a process that is mediated by the sterogenic acute regulatory protein (STAR). The importance of STAR has been illustrated by analyses of patients with lipid congenital adrenal hyperplasia, a disorder that markedly disrupts steroidogenesis (112). Although STAR plays an essential role in peripheral endocrine glands, its presence and role in the brain had been previously questioned (113). However, a number of studies have confirmed a local increase in the levels of pregnenolone and progesterone following traumatic injury in the brain and spinal cord. The expression and activity of aromatase, which synthesizes estrogen, was also found to be elevated in injured brain (114). Thus, StAR becomes an attractive pharmacological target to promote neuroprotection.

Neuropeptides. A number of peptides prevent neuronal damage in both in vitro and in vivo experiments.
The most potent are pituitary adenylate cyclase activating polypeptide (PACAP), vasointestinal peptide (VIP) and activity dependent neurotrophic factor (ADNF) which ameliorate glutamate-mediated toxicity at as low as femtomolar concentrations. The molecular mechanism of action of those neuropeptides has not been fully elucidated, however, activation of adenylate cyclase and MAP kinases, inhibition of JNK kinases and induction of some cell protecting proteins are likely to participate in this process (115, 116). PACAP also inhibits oxidative stress-induced, caspase 3 activity and attenuates interleukin-6 and chemokine release from astrocytes. Intravenous administration of PACAP prevents hippocampal pyramidal cell death in animal models of brain ischemia. VIP protects neurons by regulating cytokine release and stimulating synthesis of ADNF in glial cells. ADNF and its derivatives (ADNF-9, ADNF-14) at femtomolar concentrations exert long-lasting protective effects on neurons against oxidative stress, excitotoxicity and hypoglycemia. These peptides also increase production of anti-apoptotic transcription factor NFκB and heat shock protein hsp-60. Recently, a hybrid peptide composed of ADNF and another bioactive peptide, humanin was shown to suppress amyloid-beta toxicity and memory deficit in mice (117). A substantial evidence points also to neuroprotective properties of other neuropeptides, e.g. thyrotropin releasing hormone (TRH), neuropeptide Y, and galanin. These peptides acting on their specific receptors protect cells via presynaptic inhibition of glutamate release, and/or postsynaptically hyperpolarizing neuronal membrane (118, 119). Derivatives of TRH, novel dipeptides have multipotential actions that make them candidates for the treatment of both acute and chronic neurodegeneration (120). N-Acetylaspartylglutamate (NAAG) is the most abundant and widely distributed peptide transmitter in the mammalian nervous system. NAAG activates the metabotropic glutamate mGlu(3) receptor at presynaptic sites, inhibiting the release of neurotransmitters, including glutamate, and activates mGlu(3) receptors on glial cells, stimulating the release of neuroprotective growth factors from these cells. NAAG is inactivated by specific peptidases following its synaptic release. Novel compounds that inhibit these enzymes prolong the activity of synthetically released NAAG and have significant therapeutic efficacy in animal models of stroke, traumatic nervous system injury, amyotrophic lateral sclerosis (121).

**Neurotrophic factors.** Among the most widely studied growth factors in relation to neurodegenerative disorders is insulin-like growth factor 1 (IGF-1). IGF-I is important for fetal and postnatal development, but it also controls tissue homeostasis throughout life via the regulation of cell proliferation and apoptosis. IGF-I has multiple actions at different control points of apoptosis, including the Bcl-2 family proteins, inhibitors of caspases, and signaling of death-inducing receptors activates prosurvival PI3K/Akt or MAPK/Erk signaling pathways (127, 128). Evidence that IGF-I rescues motor neurons has led to clinical trials of human recombinant IGF-I in ALS patients (74). However, systemic delivery of human recombinant IGF-I in these trials did not lead to beneficial clinical effects in ALS patients that might have been due to the inactivation of IGF-I by binding to IGF binding proteins (IGFBPs), and/or limited delivery of IGF-I to motor neurons. The IGF analogues with low affinity for IGFBPs and analogues that are able to displace IGF-I from IGFBPs are better candidates for new clinical trials. Another possibility is to find a way of IGF-I transport without hindering the circulating and tissue-specific IGFBPs, namely IGF-I delivery based on gene therapy. Other polypeptide trophic factors, like basic fibroblast growth factor (bFGF), osteogenic protein-1 (OP-1), vascular endothelial growth factor (Veg-f), granulocyte colony stimulating factor (G-CSF), stem cell factor (SCF) and erythropoietin show beneficial effects in experimental model of acute brain ischemia and in restoring the central nervous function (129-131). However, only bFGF and erythropoietin have been admitted to further clinical trials in patients with acute stroke (132). Erythropoietin is an oxygen-dependent cytokine, which enhances red blood cell production by possible neuroprotective role of these neurosteroids in AD (122). Neurosteroids are multifunctional owning to the modulation of activity and expression of several receptors (NMDA, GABA, sigma receptors). They were demonstrated experimentally to inhibit excitotoxicity and apoptosis, so their mechanism of action in neuroprotection is under further elucidation (123-125). In order to avoid side effects of neurosteroids in the non-damaged areas in the brain and in peripheral tissues, indirect methods of increasing neurosteroid synthesis are proposed. This approach was applied mainly to ligands of peripheral-type benzodiazepine receptors, which upon activation regulate brain dysfunction by accelerating peripheral-type benzodiazepine receptor-dependent cholesterol transport into mitochondria and increasing formation of neuroactive steroids. In Alzheimer’s disease, neurosteroid biosynthesis was reported to be altered, namely there was a decrease in the level of the intermediate 22R-hydroxycholesterol, whereas this steroid was found to exert neuroprotective action against beta-amyloid neurotoxicity. Moreover, basal expression of the peripheral-type benzodiazepine receptor was up-regulated in a number of neuropathologies, including gliomas and neurodegenerative disorders, as well as in various forms of brain injury and inflammation. Hence, the use of a stable spirostenol derivative which was found to be neuroprotective, suggests that compounds developed based on critical intermediates of neurosteroid biosynthesis could offer novel means for neuroprotection (126).

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inhibition of progenitor cell apoptosis. Besides regulating proliferation and differentiation of erythroblasts, erythropoietin and its receptor seem to be involved in the central nervous system functions. It ameliorates neuronal injury because of antiapoptotic, antiinflammatory, antioxidant, angiogenic, neurogenic and neurotrophic properties (133, 134). Intracellular mechanism of beneficial effects of erythropoietin on neurons involves the activation of Jak2/Stat3 and P13/Akt pathways and stimulation of antiapoptotic protein (Bclxl) synthesis (135). Both erythropoietin and darbepoietin, a recombinant protein with erythropoiesis-stimulating activity, decrease ischemia-related brain damage and neurological deficit (131, 134, 136, 137). On the other hand, asialoerythropoietin, a neuroprotective variant of erythropoietin that lacks erythropoietic action was not effective in the R6/2 transgenic Huntington’s disease mice (138). Granulocyte-colony stimulating factor (G-CSF) is a growth factor that orchestrates the proliferation, differentiation, and survival of hematopoietic progenitor cells but there is a growing body of evidence from experimental studies suggesting that G-CSF also has important nonhematopoietic functions in the central nervous system. The neuroprotective actions of G-CSF have mainly been attributed to its anti-inflammatory and anti-apoptotic effects, induction of neurogenesis and angiogenesis. G-CSF is a potential new agent for neuroprotection but precise mechanisms of G-CSF-induced neuroprotection must be further elucidated before it enters clinical trials (139). The main obstacle for wide clinical use of trophic factors is their polypeptide structure and inability to penetrate through blood-brain barrier. To solve this problem, a chimerical brain-derived neurotrophic factor (BDNF) has been synthesized, in which the BDNF moiety was bound with monoclonal antibody against transferrin receptors expressed in the blood-brain barrier. This chimerical peptide after intravenous administration easily penetrates to the central nervous system and exerts strong protective effects on neurons in models of brain ischemia (140). Infusion of BDNF into the basal ganglia and the use of viral vectors are also tested (141-144). However, these interesting and promising approaches have to be verified in further experiments. Constructing low molecular weight peptides with neuroprotective and neurotrophic properties (e.g. cerebrolysin), which possess wider therapeutic time window could be also beneficial in neurodegeneration (145). Alternatively, the stimulation of synthesis of endogenous neuroprotective factor has been proposed as another method of treatment of neurodegenerative disorders. From this perspective, the conditional preconditioning seems to be particularly interesting. It has been found that exposure of neurons to short-lasting hypoxia or sublethal concentration of NMDA enhances expression of thireodoxin, transcription factors, like NFkB, neurotrophins and heat shock proteins (HSP), which can prevent subsequent neuronal damage resulting from lethal insult (20, 146). Alternatively, neurotrophin synthesis can be stimulated by some pharmacological agents such as vitamin D3 and some of its analogs which may ameliorate postischemic brain injury (147). In line with these data, it has been reported that some low-calcium analogues at nanomolar concentrations protect neuronal cells against NMDA- and hydroperoxide-induced damage in vitro, however, they may be less efficient in seizure-induced brain damage (148). Recently, it has been often underlined that physical exercises, cognitive stimulation and diet restriction could be a promising method to prevent or delay occurrence of neurodegenerative diseases. Evidence suggests that increased physical activity and low caloric diets could increase expression of endogenous neuroprotectants such as IGF-1 and BDNF (1, 5, 149).

**Endocannabinoids**. Evidence has accumulated over the last few years suggesting that endocannabinoid-based drugs may be potentially useful to reduce the effects of neurodegeneration. In fact, exogenous and endogenous cannabinoids were shown to exert neuroprotection in a variety of in vitro and in vivo models of neuronal injury, and the release of endocannabinoids during neuronal injury may constitute a protective response. The inhibitory effect of cannabinoids on reactive oxygen species, glutamate and tumor necrosis factor and activation of the phosphatidylinositol 3-kinase/protein kinase B pathway, induction of phosphorylation of extracellular regulated kinases and the expression of transcription factors and neurotrophins point to its multipotential activity. Dexamabinol (HU-211), a synthetic cannabinoid, is currently being assessed in clinical trials for traumatic brain injury and stroke (150, 151).

**Neuroprotective amino acids.** Some amino acids, such as taurine and kynurenic acid possess neuroprotective activity. Taurine is released from neuronal tissue upon depolarization and prevents calcium uptake, thus inhibiting excitotoxicity. Kynurenic acid is an endogenous broad-action antagonist of EAA receptors. It blocks a majority of ionotropic glutamate receptors, but preferentially acts on glycine binding to NMDA receptor complex (152). Recently, it has been demonstrated that energetic deficit decreases the activity of kynurenic acid synthesizing enzyme, and that insufficient production of this acid may participate in neurodegeneration induced by an oxidative phosphorylation inhibitor (153).

**Gene- and cell-based therapies**

Genetic approaches to the treatment of neurodegenerative disorders involve mainly constructing of viral vectors for targeted neurotrophic protein delivery as mentioned before [142]. Also viral vector-mediated gene delivery of a dopamine-synthesizing enzyme into the striatum was reported to restore local dopamine production and allowed for behavioral recovery in animal models of PD (154). Another genomic strategy, which could be beneficial in the treatment of neurodegenerative disorders, is the RNA interference (RNAi) method in silencing the expression of specific toxic genes, but it is still only experimentally studied. Recent studies have shown that cultured neurons can be efficiently transfected with siRNA and the targeted genes (e.g. p75 neurotrophin receptor, pro-apoptotic proteins from Bcl-2 family, caspases) were effectively silenced (155). Research efforts in the RNAi field aim to gain a better understanding of how its underlying machinery is orchestrated, to define the biological role of this conserved pathway, to determine how to effectively manipulate RNAi in the laboratory and to integrate all this knowledge to develop novel therapies for human dis-
eases (156, 157). It is a more promising method, since clinically relevant RNAi-mediated gene silencing in non-human primates was demonstrated (158).

Alternative strategy in combating neurodegeneration is transplantation of specific neurons to the damaged brain regions. The most advanced progress has been made in Parkinson’s disease. So far, cell replacement therapy in Parkinson’s disease (PD) has been based on the use of primary dopaminergic (DA) neuroblasts obtained from the brain of aborted human fetuses. Clinical trials show that intrastriatal DA neuron transplants can give substantial symptomatic relief in advanced PD patients but there are also several problems. First, graft survival and clinical outcome has been too variable so far, suggesting that DA neuron grafts may not be equally effective in all PD patients. Secondly, it has become clear that immune mechanisms leading to slowly developing inflammatory responses may compromise long-term graft survival and function. Third, the problems associated with the use of tissue from aborted fetuses make it necessary to develop alternative sources of cells for transplantation. Recent progress in the generation of DA neuroblasts from neural progenitors and embryonic stem cells suggests that these kinds of cells may offer more accessible, defined and standardized sources of cells for clinical transplantation in PD (159).

Animal models

Taking advantage of significant progress in molecular genetics, a number of new animal models of neurodegenerative diseases have been recently proposed. With respect to ALS, the transgenic mice harboring a human Cu/Zn superoxide dismutase 1 (SOD1) transgene containing the G93A mutation show impaired the capacity of spinal cord high-affinity glutamate uptake. Also transgenic rats expressing human SOD1 G93A with ALS-like phenotype, motor neuron degeneration in the spinal cord and reduced sensitivity to riluzole have been generated (161, 162). Another model of ALS, i.e. transgenic mice overexpressing a mutant SOD1 with a histidine to arginine substitution at position 46 (H46R), was successfully used for demonstrating of neuroprotective activity of oxidized recombinant human galectin-1 (163). For studying mechanism of Alzheimer’s disease and putative drugs, the PDAPP mice demonstrating age-dependent accumulation of amyloid beta (42)-containing plaques and decreased adult hippocampal neurogenesis seem to be an appropriate model (164). Much effort has been put into developing models of Huntington’s disease, which is caused by a polyglutamine expansion in the gene encoding the huntingtin protein (165). The rat model of this disease may be useful for testing putative drugs. For example, neuroprotective effects of intrastrial injection of M826, a reversible caspase-3 inhibitor was evidenced in this model (166). However, other models on yeast, Caenorhabditis elegans, Drosophila melanogaster and mouse have also been developed (167). An impressive progress has been made in the generation of Parkinson’s disease models. It is firmly established that the increased level of iron, MAO-B activity, oxidative stress, inflammatory processes, glutamatergic excitotoxicity, nitric oxide synthesis, abnormal protein folding and aggregation, reduced expression of trophic factors may all contribute to the mechanism of substantia nigra dopaminergic cell death. In animal models, some neurotoxins, like MPTP, 6-OHDA and met-amphetamine are commonly used as inducers of SN dopaminergic cell damage (168). Familial forms of Parkinson’s disease are associated with mutation of several genes coding for alpha-synuclein, parkin, DJ-1, UCHL1, PINK1 and LRRK2. For constructing transgenic models of Parkinson’s disease, a member of the nuclear receptor superfamily (NURR1) and a homebox transcription factor (PITX3) involved in differentiation and development of SN neurons are also employed. The genetic models have generally good construct, face, and predictive validity. However, the alpha-synuclein overexpressed, parkin knockout and DJ-1 knockout mouse phenotypes are not as profound as the Nurr 1 heterozygotes and PITX3 aphokia mice, but they may provide insight into early stages of this disease, whereas the Nurr1 heterozygotes and Pitx3 are good models to study the later stages of Parkinson’s disease (169).

CONCLUSIONS

Despite enormous progress in biological and medicinal sciences, no efficient neuroprotective drug has been designed so far. Neurochemical, genomic and proteomic studies on the mechanism of neurodegeneration provide us with a plethora of new data, however, there are still serious problems with their straightforward interpretation. Taking into account more and more complex biochemical cascades of neurodegenerative processes, it has been postulated that pharmacological multipotential or combined approaches may be most appropriate in neuroprotective strategies. Furthermore, better animal models of neurodegenerative diseases are required, and in vitro models should be based on human rather than animal neuronal cell lines, since the composition of protein targets for new drugs may show some species differences. A number of neuroprotective substances possess a narrow time-window, have dual activity (pro- or anti-apoptotic, depending on the concentration, the type of a neuronal tissue and the stage of its development), or unfavorable pharmacokinetic properties.

It appears that further improvement of the outcome of neurodegenerative disease treatments may depend not only on a better understanding of molecular mechanism of neuronal injury, but also on biopharmaceutical properties of new drugs and on properly designed clinical studies.

REFERENCES

CANCER CHEMOPREVENTIVE AGENTS-DRUGS FOR THE 21ST CENTURY?

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Abstract: After a quarter of a century of rapid advances in cancer research, the focus of oncological drug development has shifted from cytotoxic chemotherapy to rationally designed agents that target specific molecules associated with malignant cells or their environment. Carcinogenic process is driven by mutation, but there are many epigenetic variables which could be the targets of early intervention before invasion and metastasis occur. Chemoprevention is the inhibition, retardation or reversal of carcinogenic processes by pharmacological or natural agents targeting these pathways in high-risk individuals. This approach was developed more than 30 years ago and its credibility was enhanced by the positive results of clinical trials involving subjects with risk of developing breast cancer and colon tumors. So far however, not many clinical trials provided satisfying results, not only because of the lack of efficacy or side toxic effects of chemopreventive agents, but also the lack of precise biomarkers monitoring their effects. In spite of all these obstacles, the field of cancer chemoprevention is very active, not only because of its accelerating scientific base, but also because such an approach to cancer is vitally needed. New information from molecular studies has identified specific molecular targets for chemopreventive agents. These include regulatory molecules such as Nrf2, epidermal growth factor receptor kinases, components of the Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway, nuclear factor-kB, and cyclin D. The development of new drugs for the control of these targets that are both safe and effective will be important for the future of cancer chemoprevention.

Keywords: chemoprevention, COX-2 inhibitors, Nrf2, signaling pathways, transcription factors

The continuing magnitude of cancer problem clearly indicate that the more intensive approach to the prevention of this disease is the only way to eliminate the cancer burden.

Currently the basic and clinical research are driven by the elusive goal of cure the advanced disease. Given the genetic and phenotypic heterogeneity of advanced malignant lesions as they occur in individual patients, indication of specific molecular and cellular targets for the putative cure is simply impossible. Furthermore, the misperception of cancer as a disease whose most fundamental characteristic is excessive cell proliferation, has led to an over-emphasis of testing and development of cytotoxic drugs that kill cancer cells. Unfortunately, most cytotoxic drugs used in cancer chemotherapy are also highly toxic to wide spectrum of normal tissues and iatrogenic failure of the damaged organs is a frequent cause of death from cancer (1). Moreover, three-quarter of the new more specific drugs approved by U.S. FDA from 1990 through the end of 2002, did not demonstrate that they increased patient survival, but significantly increased the cost of treatment (2).

As an alternative approach, we need to consider that cancer is ultimately the end stage of a chronic disease process characterized by abnormal cell and tissue differentiation. Thus we need to focus more effort on the control of carcinogenesis rather than attempting to cure end-stage disease. This approach, called chemoprevention, was proposed already in the 1960s by Wattenberg and formal definition was introduced by M. Sporn in 1976 (3). Chemoprevention is pharmacological approach to intervention by use the natural or synthetic substances in order to arrest, reverse or delays the process of carcinogenesis. It seems that this approach toward cancer has never before held such great scientific promise and at the same time skepticism about its clinical practicality.

Despite the fact that definitive proof of principle of chemoprevention has been established in very large clinical trials including thousands of women at risk for developing breast cancer (4,5), issues of safety and liability continue to hamper the development of the field. Despite the obstacles the field of cancer chemoprevention is very active, not only because of its accelerating scientific base, but also because such an approach to cancer is vitally needed.

The advances in prevention of cardiovascular diseases dramatically reduced the death rate from myocardial infarction and stroke and provided important arguments that prevention of fatal diseases is possible. So it is time to catch up with the cardiologists.

In 2002 the chemoprevention problem was partly reviewed in Acta Poloniae Pharmaceutica (6). The present article will emphasize some other aspects and more recent progress that has occurred since then.

Conceptual basis of chemoprevention

Cancer begins through the multistage process of carcinogenesis resulting from the exposure to a wide variety of carcinogenic insults. The major stages of carcinogenesis were deduced over the past 60 years, primarily from animal model studies. These stages are termed initiation, promotion and progression (7). Tumor initiation begins when DNA in a cell or population of cells is damaged by exposure to exogenous or endogenous carcinogens. If this damage is not repaired, it can lead to genetic mutations. The responsiveness of the mutated cells to their microenvironment can be altered and may give them a growth advantages over the normal cells. In the classic two-stage carcinogenesis system in mouse skin, a low dose of initiator (polycyclic aromatic hydrocarbon, 7,12-dimethylbenz[a]anthracene, DMBA) causes permanent DNA damage (the initiating event) but does not give rise
to tumors over the lifespan of the mouse unless a tumor promoter, such as phorbol ester derivative (naturally occurring plant constituent) 12-O-tetradecanoyl-phorbol-13-acetate (TPA), is repeatedly applied (8). The tumor promotion stage is characterized by selective clonal expansion of the initiated cells, a result of TPA-induced oxidative stress, and the altered expression of genes whose products are associated with hyperproliferation, tissue remodelling and inflammation. During tumor progression, preneoplastic cells develop into tumors through a process of clonal expansion that is facilitated by progressive genomic instability and altered gene expression (Figure 1). Most animal models used in carcinogenesis research were developed before the identification of the major cancer-related genes, the recognition of the importance of host susceptibility to a carcinogenic insult, or the realization that mitogenesis and apoptosis together regulate cell number. The temporal sequence implied in this scheme is now thought to be overly simplistic and somewhat misleading. Human carcinogenesis rather than occurring in three discrete stages in a predictable order is best characterized as an accumulation of alterations in genes regulating cellular homeostasis such as oncogenes, tumor suppressor genes, caretakers gene (apoptosis-regulating genes and DNA repair genes) and stability genes (BRCA1, BLM, ATM) as well as changes in epigenome (9,10). Nonetheless, these animal models have contributed significantly to our understanding of carcinogenesis and the ways to interfere with that process. Moreover, these models allow convenient categorization of chemopreventive agents into those that can block initiation (blocking agents) and those that suppress promotion and progression (suppressing agents) (7). Early solid cancers are generally detected as intraepithelial neoplasia or carcinoma in situ, which correspond to the promotion and progression stages. Thus anti-promotion and anti-progression agents are therefore of particular clinical interest. Ultimately, such agents prevent the growth and survival of cells already committed to become malignant (secondary and tertiary chemoprevention). Chemoprevention can be applied at three levels. While secondary and tertiary chemoprevention is addressed to patients with preneoplastic lesions and cancer survivors, primary chemoprevention is thought to be applied to general healthy population and high-risk individuals and may relay to great extent on dietary intervention (11).

Proven clinical efficacy of chemoprevention and the lesson learned from its practical usage

The credibility of chemoprevention as a serious and practical approach to the control of cancer has been greatly enhanced by the results of major randomized clinical trials in the field of breast cancer. Three different agents, namely tamoxifen, raloxifene and 4-hydroxyphenylretiamide (1) have been shown effective agents for prevention of breast cancer in women of varying degrees of risk. Tamoxifen and raloxifene receptors are examples of the selective estrogen receptor modulators (SERMs) which bind to both estrogen receptors ER-α and ER-β. SERMs can act selectively in different tissues and organs either as estrogen antagonists (to suppress the undesirable cancer-promoting effects of estrogen in breast) or as estrogen agonists (to enhance the desirable growth-promoting effects of estrogen in bone).

Just released results from 20,000-women Study of Tamoxifen and Raloxifene (STAR) showed the advantages of raloxifene over tamoxifen to prevent breast cancer in high-risk postmenopausal women, providing equal cancer-preventing benefits with fewer serious side effects (12). Fenretinide is a synthetic retinoid which was shown many years ago to be a differentiating agent at many epithelial target sites in experimental animals as well as in humans (1). On the other hand, the failure of β-carotene trials provided the evidence that epidemiological data alone are not sufficient for the selection of a new agent for the major clinical chemoprevention trial. The appropriate use of a chemopreventive agent ultimately depends on the

![Figure 1. Chemoprevention of multistage carcinogenesis.](image-url)
understanding of its mechanism of action at all levels, namely at the molecular, cellular, tissue and organ levels, as well as in the animal as a whole. The trend in the field of chemoprevention has therefore been to develop new agents based on their mechanism of action.

The examples of the chemopreventive agents developed based on this approach are selective inhibitors of inducible cyclooxygenase regulating prostaglandin synthesis and involved in the inflammation. Chronic inflammation and oxidative stress contribute to experimental and human carcinogenesis (13). Two separate gene products, COX-1 and COX-2, have similar cyclooxygenase and peroxidase activities, although they are differently regulated. COX-1 is a constitutive isoform present in most tissues and mediates the synthesis of PGs required for normal physiological functions. COX-2 is not detectable in most normal tissues, but it is induced by cytokines, growth factors, oncogenes, and tumor promoters and is involved in several experimental and human cancers (14). Although the concept of the role of persistent inflammation in cancer development was proposed nearly 150 years ago by Virchov based on his observations that hematopoietic cells frequently infiltrated neoplastic tissue, the immediate relevance of this notion to modern molecular genetic was demonstrated by the finding that overexpression of the gene for COX-2 is an early and important event in colon carcinogenesis (15) and that knockout of the gene for COX-2 could suppress colon carcinogenesis in mice that were genetically predisposed to develop this condition (16).

Celecoxib, the first US FDA-approved selective COX-2 inhibitor initially developed for the treatment of adult rheumatoid arthritis and osteoarthritis, was reported to reduce the polypl burden in patients with familial adenomatous polyposis (17). The demonstration of the overexpression of COX-2 in many other forms of epithelial cancer (18) offered promise that selective COX-2 inhibitors might be used to suppress carcinogenesis in organs other than the colon.

Approximately six years after the COX-2 inhibitors were approved for use in the United States, the results of three randomized, placebo-controlled trials provided new evidence about cardiovascular risk of rofecoxib, celecoxib and valdecoxib (19,20) The public announcement of the APPROVE (Vioxx) results, which coincided with Merck’s withdrawal of rofecoxib from the market in September 2004, prompted researchers to review the cardiovascular safety results on a similar trial with COX-2 inhibitors. The results have raised the question: how did such problem arise, and how can they be prevented in the future?

COX-2 inhibitors not only lack the antiplatelet effects of aspirin; by inhibiting the production of prostacyclin, they also disable one of the primary defenses of the endothelium against platelet aggregation, hypertension and atherosclerosis (21). COX-2 inhibitors also promote an imbalance in favor of vasoconstriction. Thus the combination of reduced prostacyclin synthesis and uninhibited thromboxane synthesis in patients on long-term coxibs has been proposed as the likely explanation for coxib-related cardiovascular toxicity (22). It is also possible that it is the prolonged plasma concentrations of the coxibs that are responsible for their dangerous cardiovascular consequences.

To avoid such problem in the future, investigators must understand and respect the pharmacology underlying drug discovery. Specifically, I/ the pharmacokinetics and metabolism of the drug must be studied in relation to different dosing schedules; II/ we do not use cancer chemotherapeutic drugs as a single agents because we know that high-dose single agents induce selective survival, clonal outgrowth and ultimately recurrence. It is plausible that there is similar effect with high doses of a single chemopreventive agent, such as β-carotene (22), an effect that seems to be much less of an issue with the low-dose, multiple agent dietary interventions. At some point, we may conclude that low-to moderate dose multiple agent regimens in the prevention arena may serve us best; III/ placebo controlled, phase II chemoprevention trials must extend well beyond 3 to 6 months to at least 1 to 2 years if we are to obtain essential long-term safety data. Finally we have to remember the critical role that risk-benefit assessments play in agent development for cancer prevention.

New targets and chemopreventive agents

One of the rational and effective at least in experimental models strategies for chemoprevention is the blockade of DNA damage caused by carcinogenic insult. However clinical trials that have used exogenous antioxidants or free-radical scavengers (such as ascorbic acid and β-carotene) as chemopreventive agents to suppress mutation have been markedly unsuccessful in most cases. Therefore, a new approach to control electrophilic xenobiotics metabolites, ROS and RNO has been developed based on the intrinsic mechanisms used by the body to deactivate potentially carcinogenic molecules. This approach focuses on nuclear factor erythroid 2-related factor 2 (Nrf2), which is a leucine zipper-type transcription factor and a key enhancer of a number of genes that are cytoprotective because of the ability of their respective protein products to deactivate a large number of potentially harmful molecules. These proteins, known as phase 2 enzymes deactivate reactive electrophilic metabolites, directly destroy ROS and stabilize the oxidation and reduction potential of the cell. They include glutathione transferases, quinone reductase, epoxide hydrolase, thioredoxin, catalase, superoxide dismutase, and heme oxygenase. The new attempts to use upregulation of Nrf2 activity as a strategy for chemoprevention rely on coordinated upregulation of an entire battery of cytoprotective macromolecules that are intrinsic to the cell. This new strategy provides marked amplification of the action of chemopreventive agents, since the small molecules that induce Nrf2 activity are not themselves consumed in the deactivation of electrophiles, ROS and RNO. These inducers rather amplify physiological protective mechanisms, and this action occurs within the cell, at sites where it is needed. Nrf2 is known to regulate its various responsive cytoprotective genes through a common DNA regulatory element, anti-oxidant-response element (ARE) Nrf2 is sequestered in cytoplasm by the inhibitory protein, Keap1, until inducers react with cysteine thiol residues on Keap1, which causes a conformational change in the Keap1-Nrf2 complex and the release of Nrf2, allowing Nrf2 to activate responsive genes. Many naturally occurring and synthetic compounds such as resveratrol, curcumin, sulforaphane or oltipraz activate
the Nrf2-keap1-ARE pathways. Nrf2 can also be activated by phosphorylation, and many signal transduction cascades as those involved in mitogen-activated protein kinases, protein kinase C or phosphatidylinositol 3-kinase, can also activate Nrf2 (23). Stimulation of Nrf2-ARE signaling pathway offers unique opportunities for further development of new agents for chemoprevention. The examples are new synthetic triterpenoids agents, which can stimulate the synthesis of phase 2 enzymes at the dose levels at below 1nM (24).

While the interaction with Nrf2 may protect against DNA damage and initiation of carcinogenesis interfering or blocking the signal transduction pathway is useful for the anti-promotion/anti-progression chemoprevention strategies. The development of chemotherapeutic agents that target specific signal transduction without significant cytotoxicity opened new field also for chemoprevention. One of such approach is the inhibition of epidermal growth factor receptor (EGFR) kinase. Erlotinib, low molecular EGFR kinase inhibitor was successfully used for clinical treatment of advance breast and lung cancers and now is evaluated for the prevention of carcinogen-induced lung tumors in mice (25). The major polyphenolic constituent of green tea, (-)-epigallocatechin-3-gallate (EGCG) and very promising chemopreventive agent has been shown to modulate multiple signal transduction pathways including EGFR and MAPK cascades both in vitro and in vivo (26). Another important signal transduction pathway for chemoprevention studies is P13K-protein kinase B (Akt) pathways. Activation Akt has been shown in human bronchial preinvasive lesions (27).

As a result of research performed over the past decade it is clear that there are many fewer pathways than genes (9, 28). Thus, cross-talk of members from one that of another undoubtedly accounts for much of the redundancy, as well as diversity of responses, that can result from the surprisingly small finite number of total signal pathways. Inhibition of one pathway e.g. EGFR as in case of EGCG results in interference with multiple signaling cascades including the P13K/Akt pathway, thus blocking the activation of specific genes, DNA synthesis and proliferation (26).

Transcription factors participate in the final stages of all transduction and stress pathways by causing the up- and down-regulation of specific genes. Thus transcription factors and their associated signaling molecules also represent good targets for the development of chemopreventive agents. Transcription factor nuclear factor-xB (NF-xB) and activator-protein-1 (AP-1) were suggested as proximate links between inflammation and carcinogenesis (29, 30). The expression of COX-2 and NOS-2 is controlled by these transcription factors and selective inhibition of these enzymes is often mediated by either NF-xB or AP-1.

Several synthetic and natural compounds act in this way. Most of the COX-2 inhibitors including celecoxib and spices ingredient curcumin act through inhibition of AP-1 or NF-xB activation.

Significant inhibition of both transcription factors and COX-2 was also observed as results of rodents’ treatment with resveratrol and its analogue pterostilbene (31). Urosolic acid, naturally occurring triterpenoid inhibits NF-xB activation, which correlates with suppression of NF-xB-dependent cyclin D1, COX-2 and matrix metalloprotein 9 expressions (32). The latter gene promoter sequence similarly to cox-2 contains a binding site for NF-xB. Cyclin D1 and its relatives, cyclin D2 and D3 as key regulators of cell cycle and cellular differentiation are useful targets for chemoprevention. Aberrant expression of cyclin D1 is a frequent occurrence in bronchial preneoplasia and lung cancer and it was shown that retinoic acid can regulate the expression of all three cyclins in vitro, suggesting a potential mechanism for chemoprevention by retinoids.

The signals transducers and activators of transcription (STAT) family of transcription factors and their regulatory Janus kinases (JAKs) also play a critical role in the activities of both immune cells and tumor cells. Both sets of transcription factors represents of attractive targets for new research on its application in chemoprevention (25).

Finally, in the post genomic era of cancer biology, it is also becoming increasingly evident that epigenetic controls of gene expression play an important role in the development of cancer. Alterations in gene expression without changes in DNA coding sequences that are heritable through cell division occur throughout all stages of carcinogenesis and are involved in silencing tumor suppressor genes. DNA methylation and modification of histone protein particularly acetylation might be used for chemoprevention strategy (10).

The unique epigenetic fingerprint observed, e.g. in patients with preinvasive esophageal lesions that progressed to more advanced lesions (33) underscore the potential use of epigenetic markers in risk assessment and early detection.

**Further considerations and problems to solve**

Basically, chemoprevention is addressed to people/patients without any disease symptoms thus precise intermediate biomarkers of cancer are essential for its application. Historically, reduced cancer incidence or mortality has been required to show chemopreventive benefits. These end points make chemoprevention trials long, large, costly and hence impractical and risky for drug developers. Obviously even for clinical trials markers of carcinogenesis rather than disease are more relevant. Chemoprevention demands the change in the concept of “health” in order to consider future events not just immediate presence. Thus the development of more precise biomarkers of the earliest stages of cancer development is as important as new chemopreventive agents/drug.

Currently four categories of biomarkers are in use: I/ genetic markers (e.g. micro satellite, p53 mutation, K-ras, FHIT mutation) II/ differentiation biomarkers (squamous markers, mucine gene expression) III/ proliferation markers (retinoic acid receptor, proliferating cell nuclear antigen) IV/ efficacy markers (pharmacodynamics, indicators of oxidative state, COX-2/expressio/activity). Recent strategy of chemoprevention efficacy evaluation focuses on prevention or regression of significant precancerous lesions, intraepithelial neoplasia (IEN). Occurring in most epithelial tissue as moderate to severe dysplasia, IEN shares phenotypic and genotypic similarities with invasive disease and is on the causal pathway leading from normal tissue to cancer. The IEN was also recommended by AACR Task Force as an important target for accelerated new agent.
development (34). Development of microarray technique allowing broad transcriptional profiling adds a novel dimension to identification of molecular markers.

While the development of new synthetic chemopreventive drugs towards the new molecular targets is expected, naturally occurring substances should be profoundly evaluated. Diet-derived polyphenols, such as resveratrol are particularly attractive, since the long-proven use of their dietary sources suggests low potential for unwanted side effect. However in most preclinical studies these compounds are administered in high doses. Thus, despite the immense number of studies performed on resveratrol, the additional efficacy, bioavailability and pharmacokinetic data, particularly on nanomolar concentration are still lacking (35).

Overall cancer chemoprevention research base should also use more advanced technology like nanotechnology (36).

The inability to guarantee the long-term safety of current chemopreventive regimens is perhaps the biggest limitation to their more widespread application. Although toxic side effects are almost inevitable in the setting of chemotherapy for advanced disease, they are not acceptable for chemoprevention. One approach to consider as the conclusion from COX-2 inhibitors clinical trials would be to use intermittent ("chemoprevention window"), rather than constant, chronic dosing of chemopreventive agents. It should be also emphasize that it is unlikely that a totally safe drug regimen for chemoprevention will be ever found. As in case of other drugs evaluation of benefits vs. potential risk should decide about the implementation of chemopreventive agents.

Development of chemopreventive drugs that target mechanisms such as the inflammatory process or oxidative stress offers the possibility of immense clinical benefits not only for cancer prevention, but also other degenerative diseases where these processes play an important role in their pathogenesis. The latter should be good argument for pharmaceutical industry, which so far is reluctant to embrace cancer chemoprevention in the same way as this industry has successfully embraced the chemoprevention of cardiovascular disease.

The goal to avert invasive and metastatic malignancy by using chemoprevention is rationally, medically and economically justified. Although it is not easy the will to achieve a cancer treatment paradigm shift must be found.

REFERENCES