ABSTRACT

Design of neuroprotective drugs, such as N-methyl-D-aspartic acid (NMDA)-receptor antagonists, has been hampered by the common development of intolerable adverse effects. These adverse effects are likely caused by the high affinity these drugs have for their target, and therefore, total blockade of their normal function. Here it is demonstrated how compounds that act by an uncompetitive mechanism with a fast off-rate (so-called UFO drugs that quickly disappear from the target when no longer needed) can target pathologically active tissue. Excitotoxicity is mediated by glutamate receptors, allowing excessive calcium influx through ion channels that are integral components of these receptors in neurons and oligodendrocytes. Memantine, originally related to anti-influenza drugs, was discovered to preferentially enter excessively open NMDA-type glutamate channels and block calcium influx. Predominantly because of its fast off-rate, it does not block subsequent physiologic synaptic transmission. Memantine blockade may be illustrative of natural mechanisms by which NMDA receptor activity is modulated. A second example of tolerated drug development involves targeted S-nitrosylation of the NMDA receptor/channel complex. The addition of a nitric oxide (NO) group to cysteine residue 399 of the NR2A subunit of the NMDA receptor significantly decreases excessive channel activity. A series of bifunctional drugs (composed of memantine-NO) are being produced that target the NO group to cysteine residue 399 of the NMDA receptor. This targeting is accomplished by tethering NO to memantine, which preferentially binds to excessively activated NMDA receptor-operated channels. These new drugs can finely tune the activity of the NMDA receptor because of their dual sites of action. (Adv Stud Med. 2007;7(8):242-246)

Protecting neurons therapeutically presents several challenges. In part this is because many degenerative brain diseases involve subtle changes in signal transduction. Classically, most therapeutic drugs are designed to bind to their target with high affinity; however, in the brain these targets are intimately involved in normal function. In the face of rising (abnormal) levels of agonist for a target, such as glutamate in the case of glutamate receptors, it follows that for classical competitive inhibitors, nondiseased circuits (using low levels of glutamate) will be affected before pathologic tissue (manifesting increased levels of glutamate that will outcompete the inhibitor). This fact leads to very pronounced adverse effects. In most instances, these adverse effects are not tolerable, leading to the failure of neuroprotective drugs in many clinical trials. A new paradigm for drug design is needed. Such a paradigm has been developed, and this article will describe its application in the context of multiple sclerosis (MS).

EXCITOTOXIN-MEDIATED CENTRAL NERVOUS SYSTEM DAMAGE

Glutamate is the primary excitatory neurotransmitter of the central nervous system (CNS), and glutamate-
mediated neurotoxicity has been recognized for decades.\(^1\) Too little glutamate neurotransmission leads to drowsiness, hallucinations, and ultimately coma, whereas too much glutamate leads to cell death (excitotoxicity). There exists several glutamate receptors divided into the ionotropic and metabotropic families.\(^2\) Members of the ionotropic family include N-methyl-D-aspartic acid (NMDA), \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionate, and kainate receptors, named after glutamate-related agonists that are specific for each type of receptor. The NMDA receptor, in turn, is divided into subtypes depending on its subunit composition. Glutamate-activation of NMDA receptors results in calcium and sodium influx into the neuron, contributing to depolarization and activation of the action potential. Too much activation, however, leads to excessive calcium influx, and mediates signaling events that ultimately result in neurotoxicity. An interesting aspect of glutamate physiology is that it serves as a signaling molecule and as an important metabolic intermediate. Indeed cellular concentrations of glutamate approach 10 millimolar in neurons and glia. Thus, if a cell is injured, it is capable of releasing a significant amount of glutamate, which then serves to activate surrounding glutamate receptors and the spread of excitotoxicity. Many diseases associated at least in part with glutamate neurotoxicity, potentially including MS, are listed in the Table.

Evidence for glutamate receptor-mediated excitotoxicity in MS is derived from a variety of sources. Chronic treatment of neuronal cultures with low amounts of glutamate results in synaptic/neuronal and oligodendrocyte process damage reminiscent of neurodegenerative diseases.\(^3\) Treatment of experimental autoimmune encephalomyelitis (EAE; an experimental animal form of MS) with either NMDA or non-NMDA-type glutamate receptor antagonists ameliorates the underlying neuronal injury but not the associated inflammation.\(^4\) Interestingly, NMDA receptors also have been identified on myelin far from the oligodendrocyte cell body and are capable of fluxing significant amounts of calcium.\(^5\)

Excessive calcium influx contributes to neurotoxicity through a variety of mechanisms. Neuronal nitric oxide (NO) synthase is physically tethered to the NMDA receptor, is activated by calcium influx through the receptor-associated channel, and leads to the production of NO. This can react with superoxide anion to form peroxynitrite, which is a highly reactive and toxic molecule that does significant damage to the cell. Calcium, in sufficient quantities, also activates mitochondrial-dependent and -independent apoptosis pathways. Release of mitochondrial-associated proteins, including cytochrome c and apoptosis-inducing factor (AIF), leads to the activation of caspase-dependent and caspase-independent cascades. These pathways mediate apoptosis. Activation of p38 mitogen-activated protein kinase also can contribute to apoptotic death in this context.

**SELECTIVE BLOCKADE OF PATHOLOGIC NMDA RECEPTOR SIGNALING**

N-methyl-D-aspartic acid receptors are made up of several subunit complexes, including NR1, NR2(A-D), and sometimes NR3(A, B). Classical NMDA receptors (containing NR1 and NR2 subunits) can only be activated in the presence of both glycine and glutamate, with the glycine binding site on the extracellular surface of NR1 and the glutamate/NMDA binding site on the extracellular face of NR2. Design of a high-affinity inhibitor is straightforward using either of these sites; however, there is a simple problem. A high-affinity competitive inhibitor will always block normal activity before it blocks pathologically increased activity that leads to unacceptable side effects. However, if one designed a drug that only bound within the open channel, it potentially would preferentially work on channels that were pathologically active because they are open a greater fraction of the time. Two extreme examples of this concept

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<th>Table. Neurologic Disorders Thought to Be Mediated in Part by Excessive Glutamate Receptor Activity</th>
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include magnesium and MK-801. Magnesium is a natural blocker of the NMDA receptor-associated channel, and thus blocks calcium flux. However, its dwell time in the channel is quite short (ie, it has a very fast off-rate from the channel), on the order of a fraction of a millisecond. In contrast, MK-801 also blocks NMDA receptor-associated channels, but manifests a dwell time so long (ie, its off-rate is so slow) that it functions similarly to the high-affinity competitive inhibitors by completely blocking all activity.6 The desired properties needed would fall between these 2 extremes.

Serendipitously, we found that the antiviral drug, memantine, has just those properties.7,8 Indeed, if one performs an experiment in which NMDA is used to activate a calcium current in neuronal cultures and memantine is used to block that current, at a relatively low concentration of memantine (eg, 1 µM), blockade improves steadily as the amount of NMDA is increased (Figure 1).8 This is the opposite of what one might expect from a classical competitive antagonist and has been termed uncompetitive antagonism.9

MEMANTINE-MEDIATED NEUROPROTECTION

Since the discovery of the ability of memantine to interact with NMDA receptors, several preclinical animal studies have been performed.10 One example involves a rodent stroke model in which the middle cerebral artery was occluded for several hours.11 Memantine was administered as late as 2 or 3 hours post-infarct and reduced stroke damage by approximately 50%. Additionally, several groups have tested memantine in the EAE model, and interestingly, they found that neurologic damage was improved whereas inflammation was not affected.12,13

Several human clinical trials also have been completed, examining memantine as a potential neuroprotective agent in various neurodegenerative diseases, including Alzheimer’s disease and vascular dementia,14 as well as HIV-associated dementia.15 The conclusion to date from all of these studies is that memantine may exert at least some clinical benefit. Memantine is being tested for efficacy in human MS trials, but no data are currently available in this regard.

S-NITROSYLATION OF THE NMDA RECEPTOR:
AN ADDITIONAL NEUROPROTECTIVE STRATEGY

Earlier, the production of NO was discussed in the context of its free radical activity and ability to cause cellular damage. Proteins also can be post-translationally modified by an NO group, and in this context, the NO group is donated to a sulfhydryl or thiol group of a critical cysteine residue. This process, known as S-nitrosylation, is conceptually analogous to kinase-mediated protein phosphorylation, because it induces a functional change in the protein.

The NMDA receptor undergoes S-nitrosylation on up to 5 critical cysteine residues in its extracellular domains; the predominant reaction occurs at the cysteine residue in position 399 of the NR2A subunit.16 The consequence of S-nitrosylation of the NMDA receptor is a decrease in channel opening, thus avoiding excessive channel activity.17 Experimental identification of the cysteines critical for this process on the NMDA receptor involved the expression of wild-type and cysteine-mutated receptors in a cell type that did not express endogenous NMDA receptors. Xenopus oocytes have classically been used for this type of experiment with recombinant receptors and have made possible mutational studies of numerous different ion channels.18

First, the consensus motif for S-nitrosylation, consisting of critical amino acids that flank the reactive cysteine residue, was identified in an analogous fashion to the consensus motif for phosphorylation of proteins at threonine, serine, or tyrosine residues. Next, we found that this...
consensus motif existed around 5 cysteine residues on the NR1 or NR2A subunits of the NMDA receptor, but as mentioned earlier, the predominant inhibitory effect on channel activity was exerted by S-nitrosylation of cysteine 399 on the NR2A subunit. To prove this, as shown in Figure 2 (Panels A and B), a mutant NMDA receptor was expressed in Xenopus oocytes in which cysteine 399 was mutated to alanine. The results strikingly showed that virtually all of the effect of S-nitrosylation involved cysteine 399 because inhibition of channel activity by NO was nearly completely eliminated in the mutant. The identification of the position of S-nitrosylation, coupled with the recent identification of the crystal structure of part of the extracellular domain of NMDA receptors at atomic resolution, has allowed a structural model of the receptor to be generated demonstrating how S-nitrosylation is likely to decrease calcium influx (Figure 2, Panel C).\textsuperscript{19} Accomplishments listed in previous sentences have been important for the design of new drugs that can selectively modulate pathologic NMDA receptor function using this covalent site of S-nitrosylation.

**TARGETING NO TO THE NMDA ReCEPTOR**

If one simply administers NO to a patient, his blood pressure will drop, because one of the major physiologic roles of NO as a signaling molecule is to regulate vasodilation. A drastic drop in blood pressure could have potentially dire consequences in a patient ill from a neurologic condition, such as MS or a stroke. NO manifests other effects as well, such as inhibiting platelet aggregation. Thus, in order to limit the effect of NO to the NMDA receptor, a targeting system is needed. Memantine, given its selectivity for pathologically active NMDA receptors, was considered for this role. To this end, NO was covalently linked to memantine and used to block NMDA-induced currents and calcium influx in patch-clamp experiments to try to protect neurons from NMDA-mediated neurotoxicity. Satisfyingly, it has recently been found in preclinical studies that NO-memantine has a very strong ability to block excessive NMDA-induced ion currents and to protect neurons using such assays. Importantly, because of its dual action this effect is observed at even lower concentrations than memantine.\textsuperscript{18} By using lower concentrations of an NO-memantine drug, it is hoped that this new generation of drugs will have fewer side effects than memantine. It is expected that an NO-memantine type drug will be entering phase I clinical trials in the near future.

**CONCLUSIONS**

In MS, as in other neurodegenerative diseases, excitotoxins can activate multiple pathways leading to dysfunction, injury, and eventually death of neurons and oligodendrocytes/myelin. Elements of these cytotoxic pathways include p38 mitogen-activated protein kinase, NO/peroxynitrite, AIF, and caspases. To design drugs to combat excitotoxin-mediated neurodegeneration and avoid debilitating adverse effects, it is necessary to target pathologic activity itself. One way to accomplish this is to design an uncompetitive antagonist that has a fast off-rate (so-called UFO drugs that quickly disappear from the target when no longer needed). Memantine has...
served as the first example of this strategy. By combining compounds, such as memantine, with additional therapeutics, such as S-nitrosyl agents, one can deliver these agents specifically to diseased regions of the CNS to fine-tune activity of the target back toward normal (physiologic) conditions. Preclinical studies with proof-of-concept NO-memantine compounds have shown great promise for neuroprotection. Clinical trials of these new second-generation compounds are expected to be initiated in the near future.

**DISCUSSION**

**Q:** Has anybody checked the effect of this series of NMDA-receptor antagonist drugs on encephalopathy induced by liver abnormalities?

**Dr Lipton:** Liver-related (or hepatic) encephalopathy is thought to be, in part, mediated by an excitotoxic phenomenon. In work performed in other laboratories on animal models, memantine works for this indication. To my knowledge, however, it has not been tested in humans.

**Q:** It seems that it (hepatic encephalopathy) would be the natural model for the glutamine/glutamate excess.

**Dr Lipton:** Absolutely. In fact, you may remember one of my earlier slides where it said “metabolic diseases.” This type of disease is exactly what I was referring to.

**Q:** If this concept won the Nobel Prize, would you be the recipient?

**Dr Lipton:** I cannot answer that. I can tell you an amazing and perhaps somewhat related story, however. I was asked to give a similar talk at the Nobel Forum at the Karolinska Institute a few years ago, and at the end of the talk, amazingly, a student got up and asked, “You know, I noticed that when you give NO, Dr Lipton, you always have these molecules with it, like polyethylene glycol, which is antifreeze, or ethanol, to stabilize NO. Why do you do that? Why don’t you just give NO?” And my immediate answer was, “Well, Alfred Nobel found out the immediate answer was, “Well, Alfred Nobel found out the

2. Kew JS, Kemp JA. Ionotropic and metabotropic glutamate receptor structure and pharmacology. Psychopharmacology (Berl.) 2005;179:4-29.