New Therapeutic Strategies and Drug Candidates for Neurodegenerative Diseases

p53 and TNF-α Inhibitors, and GLP-1 Receptor Agonists

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ABSTRACT: **Owing to improving preventative, diagnostic, and therapeutic measures for cardiovascular disease and a variety of cancers, the average ages of North Americans and Europeans continue to rise. Regrettably, accompanying this increase in life span, there has been an increase in the number of individuals afflicted with age-related neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, and stroke. Although different cell types and brain areas are vulnerable among these, each disorder likely develops from activation of a common final cascade of biochemical and cellular events that eventually lead to neuronal dysfunction and death. In this regard, different triggers, including oxidative damage to DNA, the overactivation of glutamate receptors, and disruption of cellular calcium homeostasis, albeit initiated by different genetic and/or environmental factors, can instigate a cascade of intracellular events that induce apoptosis. To forestall the neurodegenerative process, we have chosen specific targets to inhibit that are at pivotal rate-limiting steps within the pathological cascade. Such targets include TNF-α, p53, and GLP-1 receptor. The cytokine TNF-α is elevated in Alzheimer's disease, Parkinson's disease, stroke, and amyotrophic lateral sclerosis. Its synthesis can be reduced via posttranscriptional mechanisms with novel analogues of the classic drug,**

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Ann. N.Y. Acad. Sci. 1035: 290–315 (2004).
doi: 10.1196/annals.1332.018

thalidomide. The intracellular protein and transcription factor, p53, is activated by the Alzheimer's disease toxic peptide, Aβ, as well as by excess glutamate and hypoxia to trigger neural cell death. It is inactivated by novel tetrahydrobenzothiazole and -oxazole analogues to rescue cells from lethal insults. Stimulation of the glucagon-like peptide-1 receptor (GLP-1R) in brain is associated with neurotrophic functions that, additionally, can protect cells against excess glutamate and other toxic insults.

KEYWORDS: **TNF-α; p53; GLP-1 receptor (GLP-1R)**

INTRODUCTION

Gaining from our expanding understanding of the medical sciences and from the discovery and introduction of new drug classes, human life span increased by more than 50% in most industrialized nations during the 20th century. Longer life is associated with both merits and costs. Hence, the continuing increase in longevity has major implications on both the societies and economies of these countries. Although hardly uncommon at the turn of the 20th century, neurodegenerative diseases were not a major cause of morbidity. In contrast, with an effective armamentarium of drugs for common infectious diseases—killers of yesteryear—together with improved preventative, diagnostic, and therapeutic measures for cardiovascular disease and a variety of cancers, degenerative disorders of the nervous system, including stroke, Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease, motor neuron diseases, and prion diseases are among the most common causes of death. They are also currently among the most debilitating illnesses and force an enormous strain on both social and healthcare budgets throughout the world.

Different brain areas are primarily affected in each of these diseases: for example cortical and striatal neurons in stroke, hippocampal and cortical neurons in AD, and substantia nigral and midbrain dopaminergic neurons in PD. However, each disorder likely develops from the activation of common final cascades of biochemical and cellular events that eventually lead to neuronal cell dysfunction and death.[1] This may involve genetic or different external triggers, such as oxidative damage to DNA, the overactivation of glutamate receptors, or the disruption of cellular calcium homeostasis, to initiate the shared cascade of intracellular events causing either acute or chronic perturbations of physiological function, cell death, and clinical manifestations (TABLE 1).[2–4] Within this shared cascade, competing biochemical pathways can steer a cell towards survival or death, even at a late stage after a potentially lethal insult. Two targets that will be discussed further and that can alter this cell survival/death balance are the GLP-1 receptor and the transcription factor p53. Very often, inflammatory reaction accompanies the pathological processes that underpin neurodegeneration and it can be initiated in numerous ways.[5–8] For example, glial activation occurs as part of a defense mechanism to facilitate tissue repair by removing unwanted cell debris. Unfortunately, such valuable actions can go awry. Activated microglial cells can produce and secrete proinflammatory cytokines, in particular TNF-α, as well as cytotoxic elements like reactive oxygen species (ROS), nitric oxide (NO), and excitatory amino acids (e.g., glutamate), and induce a self-propagating cycle of events.

Characterization of the shared biochemical cascades has provided prospective targets to intervene and potentially slow or halt the disease process. Which, if any,

TABLE 1. Examples of genetic and environmental triggers in neurodegenerative disorders

Disorder	Gene	Environmental factor
Stroke	NOTCH3, KRIT1, CST3, BRI	Smoking, lipids, calories
Alzheimer's disease	APP, presenilin-1 and -2, ApoE	Head trauma, education, physical and mental activity, calories
Parkinson's disease	α-Synuclein, parkin, DJ-1, UCHL-1, PINK1	Head trauma, toxins, calories
Amyotrophic lateral sclerosis	Cu/Zn-SOD	Autoimmune response, toxins
Huntington's disease	Huntingtin	

NOTE: PINK1, PTEN-induced putative kinase 1; UCHL-1, ubiquitin C-terminal hydrolase gene; KRIT1 encodes for Krev-1/rap1 interaction trapped 1 protein.

are pivotal and rate-limiting is a focus of ongoing research and, in part, will rely on the design, synthesis, and development of pharmacological tools to selectively bind and inhibit the function of the individual protein members within the biochemical cascade. The application of medicinal chemistry to cell biology and molecular and *in vivo* pharmacology is providing us the means of testing new therapeutic strategies to both validated and unvalidated disease targets. Our overarching focus has been to incorporate features into our initial drug design and medicinal chemistry that optimize *in vivo* targeting and provide appropriate druglike properties (pharmacokinetic and pharmacodynamic parameters) to our pharmacological tools to facilitate their rapid utility as drug candidates in proof-of-concept studies.

HALTING NEURONAL APOPTOSIS BY INACTIVATION OF p53

All differentiated cells are able to trigger their own death via activation of a genetically encoded suicide program, termed apoptosis. This biological phenomenon was originally described by Kerr and colleagues,[9] and is characterized by the presence of chromatin condensation, nuclear fragmentation, cell contraction, and dissolution into small fragments, termed apoptotic bodies, that are surrounded by plasma membrane. Phagocytosis, thereafter, removes apoptotic cells without accompanying inflammation as the integrity of the plasma membrane and organelles is maintained during the cell death process and there is no spillage of the intracellular contents. In contrast, in necrotic cell death rapid swelling and cell rupture occur and cause substantial secondary damage in surrounding tissues due to inflammation. Whereas apoptosis and necrosis differ both structurally and biochemically and hence were classified as two separate forms of cell death, both can occur side by side as a consequence of a traumatic injury with the affected cells showing a continuum of features of apoptosis and necrosis.[10,11]

From a physiological perspective, apoptosis plays a crucial role in morphogenesis during embryogenic development of the central and peripheral nervous systems,[12]

where it is pronounced when the genesis of synapses occurs. In the adult, it is also a critical mechanism for maintaining the cell number within and size of an organ or tissue that has proliferative capacity, such as the immune system or intestinal mucosa. For cells that have sustained an irreversible level of DNA damage, it represents a means of removing them from the organism and allowing their replacement by a new viable cell. While valuable in tissues with proliferative capacity by ultimately protecting the genome from accumulating excess mutations, for nervous tissue the value of this same process in the adult is questionable. Neurons that are terminally differentiated have no capacity to divide; hence, apoptosis may not be beneficial to survival, as a functioning but damaged neuron may be more useful than a dead one.

Apoptosis is a tightly regulated process that can be initiated by a variety of factors that then trigger specific biochemical pathways (FIG. 1). These have been categorized as extrinsic and intrinsic, with various interlinks. Whereas the external pathway is initiated by cell surface activation, for example, TNF-α receptor signaling through cytokine binding and activation of TNF-related apoptosis-inducing ligand (TRAIL) receptors, the intrinsic pathway is often triggered by DNA or mitochondria damage. The principal molecular components of the apoptosis program in neurons have been reviewed,[8,10,13] and broadly include Apaf-1 (apoptotic protease-activating factor 1) and proteins in the Bcl-2 and caspase families (FIG. 1).

The caspases, with 15 members in mammalian cells that can be divided into initiators (caspases 2, 8, 9, and 10) that activate effectors (caspases 3, 6, and 7), are the proteolytic enzymes responsible for mitochondrial damage, nuclear membrane breakdown, DNA fragmentation, chromatin condensation, and eventual cell death.[14] The initiator caspases possess long amino-terminal domains that contain specialized motifs that promote specific protein-protein interactions. Via these domains, as exemplified by initiator caspases 8 and 9, activation occurs by interaction with the adapter molecules, Apaf1 and FADD (Fas-associating protein with death domain). The activated initiator caspases thereafter cleave the effector ones into their active forms. Caspases, hence, provide a potential target for therapeutic intervention and significant medicinal chemistry has focused on this area.[15–17] Selective caspase inhibition has proved promising in the laboratory,[18] although some consider that they act too late in cell death pathways to have a substantial effect on long-term survival.[19] In addition, the value of this approach in brain has been limited by the poor blood-brain barrier penetration of current inhibitors[20] as these primarily are peptide-based compounds.

Another strategy would be to interrupt the apoptotic cascade upstream of caspase activation. In this regard, an important regulatory step in apoptosis occurs at mitochondrial membranes where members of the Bcl-2 family of proteins either promote (Bax and Bad) or prevent (Bcl-2 and Bcl-XL) membrane permeability transition. The ratio of specific Bcl-2 family members is hence critical as to whether cell death or protection occurs. Although signaling events acting upstream of mitochondrial changes in apoptosis are yet to be fully elucidated, the tumor suppressor protein p53, a transcription factor, clearly plays a key role[21,22] by stimulating production or mitochondrial translocation of the pro-apoptotic protein Bax and release of cytochrome *c*[23,24] among numerous other pro-apoptotic actions.[8] Whether intrinsically or extrinsically initiated, it is clear that p53 protein levels are greatly increased and the ability of p53 to bind specific DNA sequences is activated in response to cellular damage. p53 protein levels are primarily regulated posttranscriptionally, and hence

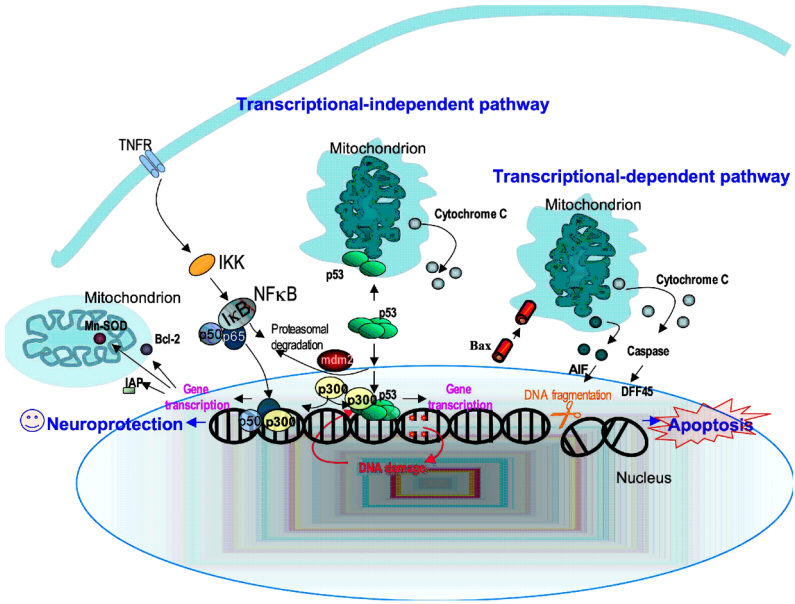

FIGURE 1. Various apoptotic stimuli, including DNA damage, induce p53 activation. This involves posttranslational modification of the p53 protein resulting in reduced proteasomal targeting by Mdm2 and, hence, increased stability and elevated p53 levels. The proapoptotic action of p53 involves its function as transcription factor inducing the synthesis and mitochondrial translocation of Bax and other p53-inducible genes, such as PUMA and Noxa. Bax induces release of apoptosis-inducing factor (AIF) and cytochrome *c* from the mitochondria. Cytochrome *c* activates caspases that cleave a series of substrates including DNA fragmentation factor (DFF45). Both AIF and DFF45 induce DNA fragmentation. Recently, mechanisms independent of p53 transcriptional activity may also be involved in p53-mediated apoptosis. p53 can translocate to the mitochondria wherein it induces release of cytochrome *c*. p53 can also block the activity of other transcription factors such as nuclear factor-κB (NFκB). NFκB is activated by downstream signaling cascades induced by tumor necrosis factor receptor (TNFR). In neurons, NFκB supports survival signaling by inducing the expression of anti-apoptotic factors, for example anti-apoptotic Bcl-2 family members, manganese superoxide dismutase (MnSOD), and inhibitors of apoptosis (IAP). p53 binds to and sequesters p300, a co-activator for various transcription factors including NFκB.

damage-induced accumulation of p53 results largely from increased stability. Phosphorylation, too, likely plays an important role in regulating its stability (such as by reducing the ability of the p53 binding protein, Mdm2, to complex and increase p53 degradation) and regulating its DNA binding activity.[25] As illustrated in FIGURE 1, the p53-dependent cascades leading to programmed cell death invoke transcription-dependent and -independent pathways with numerous regulatory feedback mechanisms to culminate eventually in cellular protection or apoptosis.

Our studies have focused on the antihelminthic (antiparasitic) compound, pifithrin-α (PFTα), as a pharmacophore for drug design (FIG. 2) because the agent

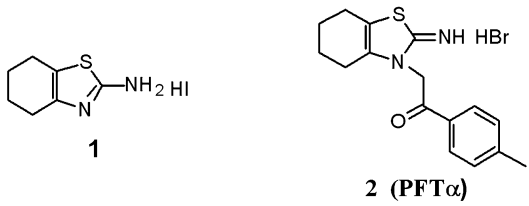

FIGURE 2. Chemical structures of the p53 inactivators, PFTα and precursor (compounds 2 and 1, respectively).

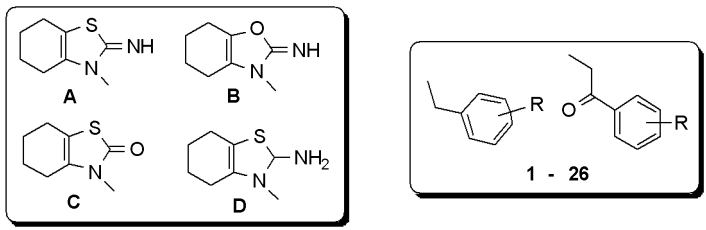

FIGURE 3. Heterocycle and *N*-substituent modifications to create PFTα analogues.

was recently reported to inhibit p53-mediated transcription.[26] Our initial studies demonstrated that compound 2, but not its precursor (compound 1) (FIG. 2), was effective in protecting cultured primary hippocampal neurons against death induced by the DNA-damaging topoisomerase I and II inhibitors, camptothecin and etoposide, respectively.[27] These agents induce neuronal apoptosis via a mechanism that involves p53 induction and caspase activation,[28,29] which parenthetically were suppressed in the presence of compound 2. Compound 2, additionally, protected neuronal cells against fatality induced by glutamate and Aβ,[27] which similarly induce p53-mediated neuronal death.[1,30] However, it was ineffective in preventing cell death caused by trophic factor withdrawal,[27] which is known to kill cells in a p53-independent manner.[31,32] These studies confirmed the activity of compound 2 as a p53 inactivator and hence provided a lead compound for medicinal chemistry.

The inactivity of the PFTα precursor, compound 1, indicates that the *N*-substituent group of compound 2 is critical for p53 inactivation. Modifications were therefore initially made to this moiety to elucidate structure-activity relationships for neuroprotective activity. Analogues were synthesized to assess the importance of the carbonyl and aryl groups, together with substitution of electron-donating and electron-withdrawing moieties on the latter.[33] In addition, heterocyclic modifications also were undertaken to optimize the pharmacophore for greatest neuroprotective activity (FIG. 3). Numerous novel analogues were synthesized, possessing calculated log D values (clog D: octanol/water partition coefficient at physiological pH) that primarily ranged between 0 to 2.5, in line with the Lipinski "Rule of 5"[34] to optimize absorption and brain delivery.[35] The log D value provides an indication of blood-

brain barrier penetrability and the upper value is in line with that of the classic CNS active drug valium. Illustrated in FIGURE 4 are examples, among many modifications, which result in altered activity. Interestingly, and as depicted by B5 vs. A5 (FIG. 4), replacement of the heterocycle S by an O (resulting in tetrahydrobenzoxazoles rather than -thiazoles) resulted in greater potency (as determined by a lower EC_{50} value for protecting PC12 cells from camptothecin-induced apoptosis). Modifications in the *N*-substituent group of the parent compound, such as removal of the carbonyl function (depicted by A19 vs. A5, FIG. 4) resulted in loss of activity. However, incorporation of a halogen within aryl function (depicted by A15 and A17 vs. A5) reinstated biological activity and, indeed, improved it depending on the position of substitution. Likewise, as depicted by A7, A8, and A9 vs. A5 (FIG. 4), replacement of an aryl methyl group by an electron-donating methoxy dramatically altered biological activity depending on the position of substitution.[33] In this manner, a wide variety of compounds of varying p53 inhibitory potency were synthesized with physicochemical characteristics to either augment or restrict brain uptake for neurodegenerative and systemic diseases, respectively, together with modifications to protect against plasma lability.[33]

The mechanisms underpinning p53 inactivation remain to be fully elucidated. PFTα and active analogues lowered the basal level of p53 DNA-binding activity in resting, unchallenged neuronal cells in culture, without affecting basal levels of p53-responsive genes, as exemplified by Bax.[27] Consequent to an insult with Aβ or camptothecin, however, PFTα markedly suppressed p53 induction, phosphorylation, the induction of Bax, and downstream events associated with apoptosis, such as stabilizing mitochondrial function and suppressing caspase activation.[27,36]

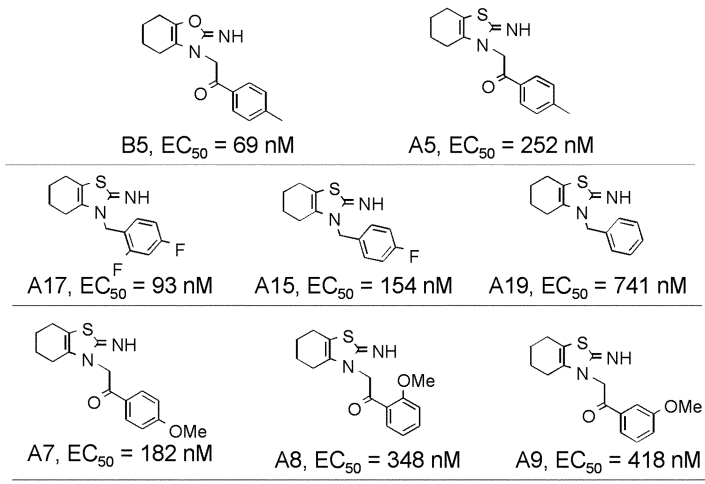

FIGURE 4. PFTα analogues: examples of structure-activity relations. EC_{50} values were determined in PC12 cells challenged with a lethal dose of camptothecin, which induces cell death via a p53-dependent mechanism (EC_{50} represents the concentration of compound required to protect 50% of cells from death).[33,117]

Preclinical studies have focused on the selection of analogues as candidates for potential clinical development. In this regard, "proof of concept" preclinical efficacy studies have been undertaken in a variety of models, exemplified by stroke and PD, to assess the potential of transient p53 inactivation.[27,33,36,37] Such studies, together with classic pharmacokinetic and pharmacodynamic studies in rodent models, aid in compound selection to determine which of the numerous agents available should be developed as experimental drug candidates. A potentially serious adverse action of this class of compounds is the risk that they may induce or increase the risk of tumors. p53 knockout mice have an increased incidence of cancer. In contrast, its overactivation induces premature aging-associated phenotypes in mice.[38,39] Consequently, transient and relatively short-term inhibition of p53 in the treatment of acute catastrophic insults, exemplified by stroke or head trauma, could prove efficacious, as could long-term inhibition of induced p53 rather than constitutive, basal levels during a devastating, longer term disorder, such as PD and AD. Only classical toxicological studies can define whether risk/benefit ratios are appropriate to support the strategy in specific indications.

Most research has focused on stroke, utilizing the classical middle cerebral artery occlusion (MCAO) model in the mouse, wherein focal ischemia is induced by either transient or permanent occlusion. An ischemic infarct results in damage to the ipsilateral cerebral cortex and striatum, whose size can be measured at a fixed time thereafter (e.g., 24 or 48 h) by staining brain sections with a dye taken up by functioning mitochondrion.[27,33,36] In both stroke models, p53 inhibition lowered infarct volume[33] (as achieved with PFTα, FIG. 5), with reductions occurring in the ischemic penumbra as opposed to the central core.[36] A concentration-dependent effect was

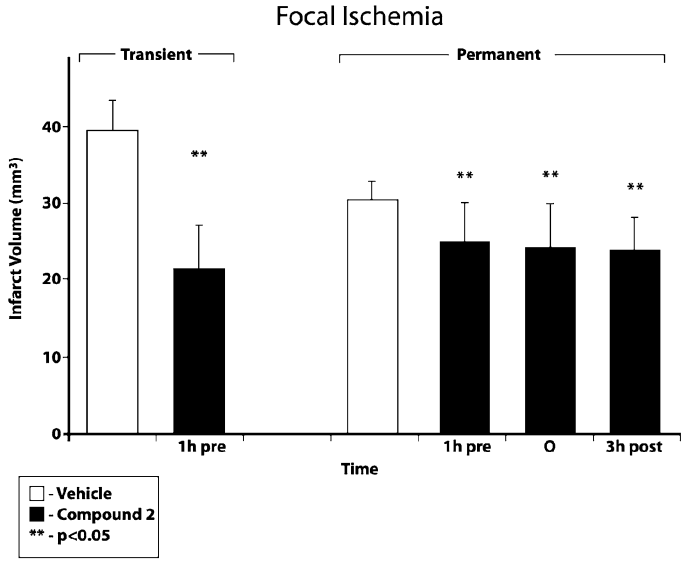

FIGURE 5. Protective effect of PFTα (compound 2) (2 mg/kg i.p.) in mice before and after focal ischemia. Infarct volume was assessed at 24 h.[33]

evident, which demonstrated a classical inverted U-shaped curve, with a loss of protective activity occurring at high doses.[36] Reduced infarct size was associated with lower disability scores and could be achieved within a therapeutic window of some 4 h after MCAO. Administration of p53 inactivators in stroke, similar to cell culture studies assessing other apoptotic insults, was associated with lowered levels of both induced and phosphorylated p53, a reduced expression of its targeted genes $p21^{WAF}$ and Bax, and less activation of caspases in the infarct tissue, compared to untreated animals after MCAO.[36] Of particular interest, immunohistochemical staining for p53 and $p21^{WAF}$ within the ischemic penumbra of PFTα treated and untreated mice demonstrated a similar number of p53-positive cells in each, p53 localization in the cytoplasm in the former as opposed to the nucleus for the latter, and a lack of $p21^{WAF}$ staining in the former.[36] This is indicative of PFTα and analogues acting at the level of p53 translocation into the nucleus and the prevention of its binding to specific DNA sites. Likewise, studies in cortical synaptosomes have demonstrated that p53 has a role in synaptic dysfunction and loss that occurs during the early stages of the neuronal death process, which can be protected by PFTα that blocks the translocation of p53 to mitochondria.[40]

Similarly, p53 inactivators have been employed in a mouse model of seizure-induced brain damage, in which intrahippocampal administration of the excitotoxic kainate induces seizures and selective degeneration of pyramidal neurons in regions CA1 and CA3 of the hippocampus. Here too, PFTα provided neuroprotection,[27] as well as in a classic mouse model of PD, wherein selective dopaminergic cell death was induced by the mitochondrial poison, MPTP.[37] Whereas the mechanisms whereby PFTα and analogues inhibit p53 transcriptional-dependent and -independent activities remains largely unknown, they reliably inhibit activation of p53-responsive genes in neuronal cells in both cell culture and *in vivo*.[27,33,36,37,40] The specificity of these agents is confirmed by their inability to protect cells lacking p53 or tumor cells with p53 mutations against apoptosis.[26,40] The factors that cause p53-mediated apoptosis in neurodegenerative diseases, such as PD and AD, are not fully understood, but likely involve common cascades, albeit initiated by different environmental as well as genetic factors, leading to mitochondrial impairment and increased oxidative stress. Thus, our data, which show that chemical inhibitors of p53 can prevent apoptosis of different vulnerable neurons in a host of insults in cell culture as well as in animal models, and yet preserve motor function, suggest that their use, if sufficiently well tolerated, might delay or halt the neurodegenerative processes associated with a variety of acute and chronic insults.

INHIBITING THE INFLAMMATORY PROCESS ASSOCIATED WITH NEURODEGENERATION

Inflammatory processes play a critical role in promoting the degenerative processes in a wide variety of neurodegenerative disorders, including AD, PD, ALS, and MS.[5,41–43] Although inflammation represents a first line of defense against injury and infection, a disproportionate inflammatory response can cause additional injury to neural cells. Indeed, many neurodegenerative disorders are associated with the accumulation of abnormal protein assemblies, exemplified by Aβ in AD and α-synuclein in PD. There is increasing support to suggest that such assemblies can

instigate cellular stress and neuro-inflammation, to which neurons have a relatively low tolerance, thus potentially initiating a self-propagating cycle of autodestructive immune and inflammatory processes that hamper the recovery of neurological function at sites of inflammation or that even intensify neuronal injury.[44]

The characteristic markers of chronic inflammation are associated with most neurodegenerative disorders,[5,6] amongst which there is upregulation of the pro-inflammatory cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6), and TNF-α, as well as the prostaglandin generating cyclooxygenases, COX-1 and COX-2.[7,44–46] In AD, the impact of these inflammatory processes is now well accepted. In PD, however, although inflammatory markers in the substantia nigra were reported long ago[5] and the high number of microglia associated with this area may underpin its vulnerability,[47] the impact of these processes remains a matter of debate. Nevertheless, anti-inflammatory therapy may represent a promising therapeutic intervention.[43,48] The question remains as to the choice of target.

The potential that COX-1 and COX-2 inhibitors have demonstrated in cell culture and animal models has, unfortunately, not yet been realized in clinical AD trials focused on disease progression. Although the results have been disappointing,[49] the use of non-steroidal anti-inflammatory agents has been associated with a lower risk of the disease.[6,50] This has stimulated interest in the potential value of inhibiting other specific cytokines. In this regard, the role of IL-1β in neuroinflammation and AD has recently been examined,[51] as has TNF-α.[52] Both IL-1β and TNF-α are expressed in microglia around developing amyloid plaques in brain cells[53,54] and have been shown to stimulate APP turnover into its pathological Aβ form.[55–57] Indeed, these cytokines are potent stimulators of γ-secretase, resulting in the increased production of Aβ through a pathway involving the c-Jun N-terminal kinase (JNK)–dependent MAPK pathway[58] and, additionally, upregulate the APP expression of cells.[59–61] Furthermore, astrocytes and microglia, both representing key components of the cellular environment surrounding neurons, are non-neuronal sources of APP that are activated in the presence of amyloid plaques[62,63] as well as by fibrillar Aβ, through a cell surface receptor complex, to further increase their production of TNF-α and IL-1β.[64,65]

These same cytokines represent a useful target in PD, where there is evidence of the upregulation of TNF-α, in particular, in both brain and CSF.[66–68] In addition, an earlier onset of sporadic PD has been reported in Japanese patients with a polymorphism in the promotor region of the TNF-α gene.[69] The genetic ablation of TNF-α in mice provides them resistance to MPTP toxicity[70] as well as to EAE.[71] Finally, overexpression of TNF-α is found in brain trauma and cerebral ischemia,[46,72] multiple sclerosis,[73] as well as ALS.[74] Clearly, cytokines have a beneficial role, as evidenced in their reported neuroprotective actions in β-amyloid toxicity,[75] focal cerebral ischemia, and epileptic seizures.[76] Their actions are concentration- and time-dependent, and a reduction of their levels towards basal ones could prove to be efficacious in several neurodegenerative conditions.

In our continuing effort to design and develop agents of potential clinical value, we focused on the pharmacophore of thalidomide to synthesize novel and pharmacologically useful agents. Thalidomide (*N*-α-phthalimidoglutarimide) (FIG. 6) is a glutamic acid derivative that was introduced onto the market as a sedative hypnotic in 1956. It was withdrawn from the market in 1961 due to the development of severe congenital abnormalities in babies born to mothers using it for morning sick-

ness.[77,78] Interest in the agent was rekindled after thalidomide was found clinically effective in the treatment of erythema nodosum leprosum (ENL)[79] and in the treatment of HIV wasting syndrome and various cancers.[80] Mechanistic studies of its ENL activity demonstrated an anti–TNF-α action. Specifically, thalidomide enhances the degradation of TNF-α RNA and thereby lowers TNF-α synthesis and secretion.[81,82] Further studies have defined it as a co-stimulator of both CD8+ and CD4+ T cells,[83] an inhibitor of angiogenesis[84] via its inhibitory actions on basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), and an inhibitor of NFκB. However, it is the action of thalidomide on TNF-α that has provided our

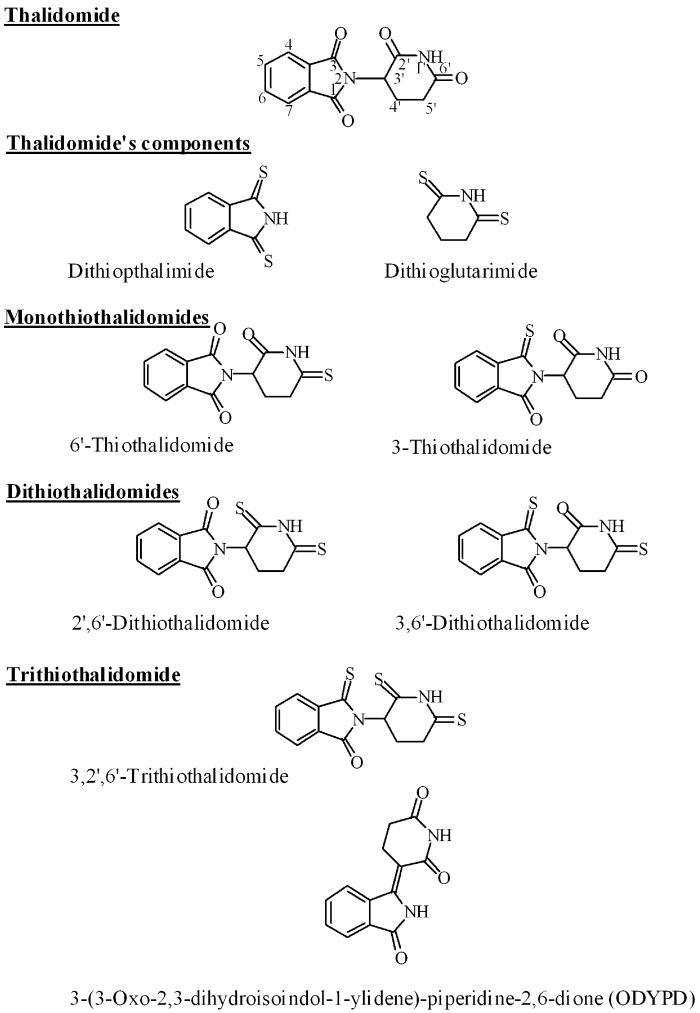

FIGURE 6. Chemical structures of analogues of 3-phthalimidoglutarimide: thalidomide.

initial focus. Clearly, this critical cytokine has numerous fundamental roles beyond neurodegeneration, as its overproduction exacerbates inflammatory and autoimmune responses in a wide number of systemic diseases, including rheumatoid arthritis, ENL, septic shock, graft-versus-host disease, Crohn's disease, and AIDS.

The mechanisms underlying thalidomide's diverse actions, together with identification of the active species (since it generates numerous metabolites) remain an area of intense research. Seldom has such a simple molecule had such a controversial history and held so much therapeutic promise. Our focus was the design of agents with improved TNF-α inhibitory potency plus a balanced lipohilicity to combine a reasonable solubility in the aqueous phase of plasma with the ability to penetrate biological membranes readily, such as the gastrointestinal tract and blood-brain barrier. Prior research had primarily focused on exploring the structural modification of the phthaloyl ring or glutarimide ring of thalidomide, including *N*-phthaloyl 3-amino-3-arylpropionic acid derivatives, amino-phthaloyl substituted and tetrafluorophthaloyl substituted analogues of thalidomide, and *N*-substituted phthalimides with a simplified glutarimide moiety.[82,85–88] In addition, as the anti-angiogenic property of thalidomide has been attributed to its hydroxylated metabolites,[89,90] the open-ring metabolites have also been synthesized.[89,91]

To discover novel isosteric analogues of thalidomide, we focused on the carbonyl moiety and synthesized a novel series of thiothalidomides and analogues (exemplified by compounds in FIG. 6). Their TNF-α inhibitory activity was thereafter assessed in human peripheral blood mononuclear cells (PBMCs) that were stimulated with lipopolysaccharide (LPS) to elevate levels of constitutively expressed TNF-α. As shown in TABLE 2 and FIGURE 7, thalidomide was essentially devoid of activity at 30 μM, requiring a concentration of ~200 μM to induce a 50% inhibition (IC_{50}) of TNF-α secretion. By comparison, the monothiothalidomides, 3-thiothalidomide and 6′-thiothalidomide demonstrated albeit minor but nevertheless significant activity

TABLE 2. Thalidomide and analogue-induced inhibition of LPS-stimulated TNF-α production in PBMCs and cell viability

Compound	IC_{50} (μM)	Percent inhibition at 30 μM	Percent cell viability at 30 μM	Clog D value (lipophilicity)
Thalidomide	~200	None	100	–0.83
6′-Thiothalidomide	>30	31	100	–0.57
3-Thiothalidomide	>30	23	94	–0.57
2′,6′-Dithiothalidomide	20	52	69	–0.56
3,6′-Dithiothalidomide	11	61	100	–0.56
3,2′,6′-Trithiothalidomide	6	79	94	–0.30
Dithioglutarimide	8	75	100	–0.48
Dithiophthalimide	3	95	54	+0.27
ODYPD	>30	37	100	+0.29

NOTE: Clog D value: measure of solubility in aqueous/lipophilic environment, with minus value representing aqueous solubility and plus value representing lipophilic solubility.[35]

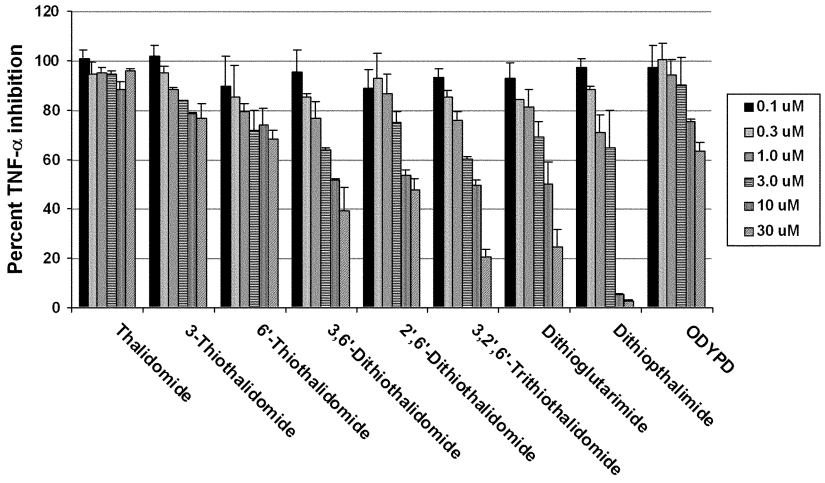

FIGURE 7. Concentration-dependent activity of thalidomide and analogues to lower LPS-stimulated TNF-α levels in human PBMCs (Mean±SEM, $N \geq 3$).

at 30 μM, inducing a 23% and a 31% inhibition of TNF-α secretion, respectively, without a loss of cell viability.

In contrast, the dithiothalidomides, 3,6′-dithiothalidomide and 2′,6′-dithiothalidomide, proved to be yet more potent (FIG. 7). They induced a 61% and 53% TNF-α inhibition at 30 μM, albeit with a slight loss of cell viability for only the latter compound, and possessed IC_{50} values of 19.5 μM and 11.5 μM, respectively. In line with the illustrated augmented TNF-α inhibitory action with successive isoteric carbonyl to thiocarbonyl replacement, the trithiothalidomide, 3,2′,6′-trithiothalidomide, inhibited TNF-α production with an IC_{50} of 6.2 μM without any accompanying toxicity. As shown in FIGURE 7, an increasing concentration-dependent inhibition was achieved that induced a 79% reduction in secreted TNF-α levels at 30 μM. Hence, replacement of successive carbonyl groups with a thiocarbonyl group led to an increased inhibitory activity compared to thalidomide, of up to 30-fold for 3,2′,6′-trithiothalidomide, that was unassociated with toxicity, but coupled to an elevated lipid solubility, as assessed by clog D values (TABLE 2), to aid in driving the compound into the brain.

As thalidomide comprises of two distinct chemical moieties, specifically adjoining glutarimide and phthalimide rings, dithioglutarimide and dithiophthalimide were therefore synthesized and evaluated for biological activity against TNF-α. Interestingly, both proved to be substantially more potent than thalidomide; inducing a dramatic inhibition of 75% and 95% at a 30 μM concentration, respectively, albeit with some toxicity for the latter. However, such toxicity was not found at the IC_{50} concentrations, which were 8 and 3 μM for dithioglutarimide and dithiophthalimide, respectively. Furthermore, as the pthalimide and glutarimide rings are conjoined at the 2- and 3′-positions, modification of this was undertaken by the synthesis of ODYPD, whose TNF-α inhibition was more potent than thalidomide (37% vs. no

inhibition at 30 μM, respectively), indicating that thionation of this as well as other thalidomide-like backbones could provide increased TNF-α activity.

Additional studies were undertaken to elucidate the mechanism underpinning the activity of the thalidomide analogues on TNF-α. TNF-α and other cytokines and protooncogenes are known to be regulated at the posttranscriptional level.[92,93] Seeing that thalidomide has been reported to act via the 3′-UTR of TNF-α mRNA that contains an AU-rich area,[81] specific studies were undertaken to assess this with dithioglutarimide and 3,2′,6′-trithiothalidomide. In this regard, a cell-based assay consisting of two stably transfected cell lines derived from the mouse macrophage line (RAW264.7) was used. One line expressed a luciferase reporter construct without any UTR sequences, whereas the other expressed a luciferase reporter construct with the entire 3′-UTR of human TNF-α inserted directly downstream of the luciferase coding region. As illustrated in FIGURE 8, concentration-dependent reductions in the ratio of the luciferase plus 3′-UTR versus the luciferase minus 3′-UTR were found that paralleled reductions in PBMC TNF-α levels. This suggests translational regulation as an underlying mechanism, whereby TNF-α mRNA is destabilized in the presence of the compounds to lower newly synthesized TNF-α protein levels. Current studies are assessing whether or not the described reductions translate into *in vivo* models and are efficacious in specific neurodegenerative diseases.

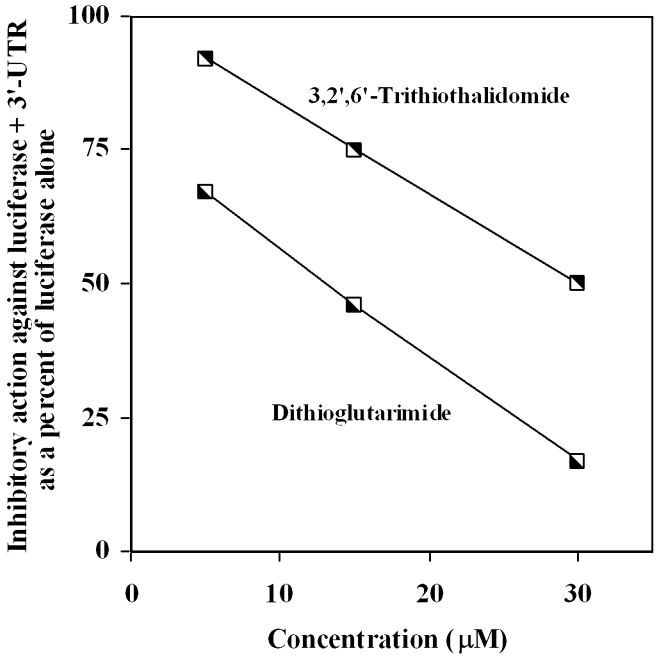

FIGURE 8. The inhibitory actions of dithioglutarimide and 3,2′,6′-trithiothalidomide in mouse macrophage cells (RAW264.7) expressing a luciferase reporter element together with the 3′-UTR of human TNF-α compared to cells lacking the 3′-UTR.

Interestingly, TNF-α inhibition is a validated therapeutic strategy for the treatment of adult and juvenile rheumatoid arthritis and other systemic indications involving inflammatory processes, with the approval of the prescription medications, Enbrel (Etanercept, Amgen/Wyeth) and Remicade (infliximab, Centocor/Schering-Plough). These medications, however, are large macromolecules that minimally traverse biological barriers, require subcutaneous and intravenous injection, respectively, for administration and have negligible brain penetration—thereby precluding their utility in neurodegenerative disorders. In contrast, thalidomide analogues are orally bioavailable and, if well tolerated in toxicological studies and not administered during pregnancy, may be of potential in a wide spectrum of diseases.

GLP-1 RECEPTOR ANALOGUES AS NEUROPROTECTIVE AGENTS

GLP-1 is an endogenous insulinotropic peptide that controls plasma glucose levels via its action on the pancreas; specifically, via the G-protein coupled GLP-1 receptor (GLP-1R) (FIG. 9). The peptide derives from the posttranslational modification of proglucagon and is released from intestinal L cells in response to the presence of food in the gastrointestinal tract.[94] It has an array of reported physiological actions that include glucose-dependent stimulation of insulin secretion and inhibition of glucagon secretion,[95] the inhibition of small bowel motility as well as gastric emptying, and a reduction of hunger.[94–96] GLP-1 additionally acts as a trophic agent, inducing pancreatic β-cell proliferation and neogenesis as well as an inhibition of β-cell apoptosis.[97,98] As a consequence, GLP-1 is an important regulator of β-cell mass, which, together with its other physiological actions, has spurred the development of GLP-1R agonists as a treatment strategy for type 2 diabetes. An advantage of this approach is that many of its effects are glucose-dependent and hence its use is associated with a low risk of hypoglycemia. A disadvantage is that, like most endogenous peptides, GLP-1 is short-lived. It is rapidly degraded by the enzyme, dipeptidyl peptidase IV (DPPIV), and then cleared by the kidney. Hence, its biological activity is short-lived in humans and rodents.[99,100] This, however, has been overcome by both peptide modification, to provide DPPIV-resistant analogues such as exendin-4 (FIG. 9), as well as by the use of DPPIV inhibitors.[94,97,100]

Although predominantly localized to pancreatic islets, numerous reports have documented GLP-1R expression in both the rodent[101,102] and human brain.[103,104] Whether or not GLP-1 is produced by neural cells, though, remains to be determined. It is clear, however, that GLP-1 present in the bloodstream can enter brain.[105]

GLP-1 (7-36 amide) **HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR.**
Exendin-4 (1-39) **HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS**

FIGURE 9. Amino acid sequences of GLP-1 and its long-acting analogues, exendin-4. GLP-1 is cleaved at the amino terminal by DPPIV (*light gray* amino acids) to end its biological action. The replacement of HA by HG dramatically reduces this. *Underlined* amino acids differentiate GLP-1 and exendin-4, and the 9 amino acid carboxy terminal tail associated with the latter provides it higher potency at the GLP-1R.[100,118,119]

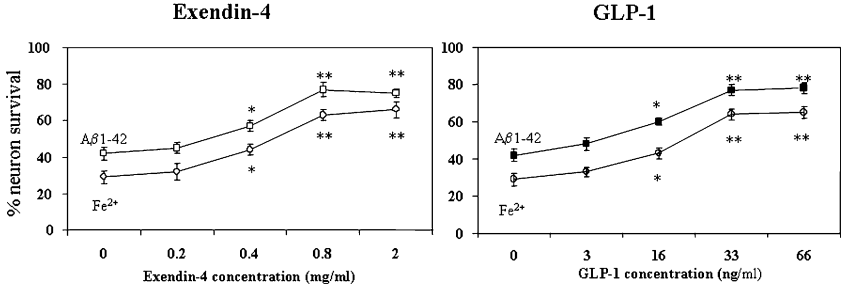

FIGURE 10. GLP-1R stimulation by either GLP-1 or its long-acting analogue, exendin-4, dose-dependently protected cultured rat primary hippocampal cells against apoptosis induced by $A\beta_{1\text{-}42}$ (2 mM) or iron (1 mM) (*P<.05, **P<.01).[108]

In light of the described trophic action of GLP-1R stimulation on β cells, together with its coupling to the cyclic AMP (cAMP) second messenger pathway, increases in which are well documented to be associated with neuroprotection, we characterized the action of GLP-1 analogues on neuronal cells both in cell culture and animal studies.[106–108]

Cell culture studies with both rat pheochromocytoma (PC12) and rat primary hippocampal cells established that the cells express GLP-1 receptors and that GLP-1R activation stimulated adenylyl cyclase, leading to an increase in intracellular cAMP in a manner similar to pancreatic β cells.[106] In addition, GLP-1 together with long-acting analogues induced differentiation in PC12 cells in a manner similar to nerve growth factor (NGF), which was reversed by co-incubation with a selective GLP-1R antagonist. Furthermore, GLP-1 analogues enhanced NGF-initiated differentiation and partly rescued degenerating cells from NGF-mediated withdrawal, in the absence of cellular dysfunction or toxicity.[106] Competitive binding studies demonstrated that the binding affinity of GLP-1 for receptors on hippocampal neurons (IC_{50} of 14 nM) was similar to pancreatic β cells. In addition, GLP-1 analogues provided complete protection against apoptotic cell death induced by glutamate neurotoxicity in cultured hippocampal neurons,[107] as has been shown with other neurotrophic factors.[109,110] Likewise, and as illustrated in FIGURE 10, hippocampal cells were protected against cell death induced by $A\beta_{1\text{-}42}$ as well as from oxidative stress and membrane lipid peroxidation caused by iron.[108] Together, these data suggest that GLP-1 agonists may play a significant role in protecting neurons against several types of brain injury, including excitotoxic and oxidative damage.

Whereas much is known about the cellular signaling pathways that occur following GLP-1R stimulation in pancreatic β cells,[97,98] as yet, less has been elucidated in neuronal cells. Our findings implicate participation of phosphatidylinositol-3 kinase (PI3-kinase) and extracellular signal–regulated mitogen-activated protein kinase (ERK MAPK)–dependent pathways in GLP-1–mediated neurite outgrowth and neuroprotection, with additional involvement of protein kinase-A (PKA) signaling.[106,108,111,112] As illustrated in FIGURE 11, stimulation of these and related pathways can divert signaling away from apoptosis towards cell survival.

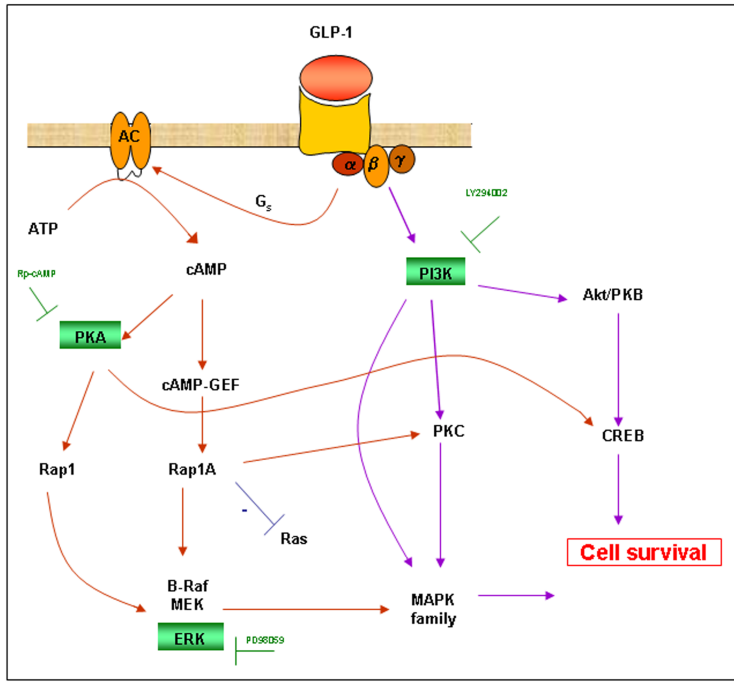

FIGURE 11. Putative signaling pathways stimulated by GLP1-R binding and activation supporting cell survival.GLP-1 action is mediated following binding to a specific G-protein coupled GLP-1R that is coupled positively to the adenylate cyclase (AC) system. Ligand activation of the Gα subunit of the GLP-1R stimulates AC, which leads to an increase in intracellular cAMP and activation of PKA. cAMP activates a GTPase of the Ras superfamily, Rap1, following PKA-dependent phosphorylation. However, cAMP also activates multiple intracellular signaling pathways independently of its activation of PKA. One such pathway has been postulated in β cells, which involves two types of cAMP-GEFs (which are activated by binding cAMP and result in activation of Rap1A). Rap1A subsequently inhibits Ras, but activates PKC and B-Raf, both of which result in activation of MAPKs. The Gβγ dimer activates PI3K, which subsequently activates MAPKs by a PKC-dependent (*dark gray arrows*) or independent (*light gray arrows*) mechanism. Inhibition of PI3K (with LY294002) or ERK/MAPK (with PD98059) results in limited GLP-1 stimulated neurite outgrowth,[106] whereas simultaneous inhibition of both pathways blocks differentiation. GLP-1R activation has been shown to protect against apoptotic insult in a number of different cell types, which can be abolished by Rp-cAMP (a cAMP-dependent inhibitor of PKA), which indicates cAMP is a positive mediator in the prevention of apoptosis. Likewise, the PI3K inhibitor with LY294002 inhibits the anti-apoptotic activity of GLP-1, which indicates this effect is at least in part, regulated in a PI3K-dependent manner.[106] Interestingly, inhibition of MAPK/ERK with PD98059 does not inhibit the protective properties of GLP-1, suggesting that MAP/ERK signaling is not critically involved in cell survival in insulinoma cells. Such studies have not yet been addressed in neuronal cells and there is insufficient evidence to rule out a role for all MAPK pathways in the anti-apoptotic action of GLP-1. It seems likely that the protective properties of GLP-1 are mediated through PI3K-induced phosphorylation and activation of Akt, with subsequent CREB-stimulated gene expression of nuclear targets.

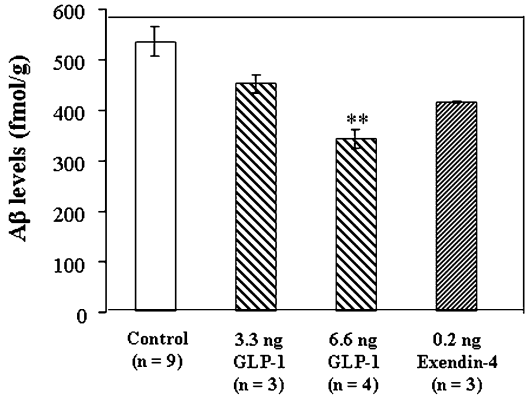

FIGURE 12. GLP-1R stimulation lowered brain levels of Aβ in mice following a single administration of either GLP1 or its long-acting analogue, exendin-4 (Aβ levels were assayed by sandwich ELISA of whole brain homogenates 48 h after administration, using a mouse-specific antibody, $^{**}P<.01$).[108]

In light of the protective action of GLP-1R stimulation against toxic insults in neuronal cells, the physiological relevance of GLP-1 analogues was assessed in rodents. Specifically, the ability of GLP-1 to modify the synthesis/processing of APP and Aβ was evaluated, together with its potential to protect neurons against apoptosis in a classic model involving the ablation of presynaptic cholinergic neurons that mimics the cholinergic deficit associated with early AD. As shown in FIGURE 12, GLP-1 analogues lower Aβ in normal mouse brain by some 20%. The mechanism(s) underpinning this activity remains to be elucidated, as Aβ levels can be altered via numerous processes.[57,113] In cell culture studies, GLP-1 analogues lowered both secreted and cellular APP levels,[108] suggesting a reduction in APP synthesis, in a manner analogous to that reported for the AD experimental drug, phenserine,[114] which acts posttranscriptionally to destabilize APP mRNA at the level of its 5′-untranslated region and, consequently, lower APP protein synthesis. This, in turn, can lower Aβ levels. Alternatively, reductions in APP and Aβ could be mediated via cAMP, which, as discussed, is dramatically elevated by GLP-1R activation, or by direct action on α-, β-, or γ-secretases, which has not yet been assessed.

In a well-established rodent model of cholinergic neurodegeneration,[115] we have shown complete amelioration of an ibotenic acid–induced cholinergic marker deficit following infusion of GLP-1. As illustrated in FIGURE 13, a partial unilateral ibotenic acid basal nucleus lesion was followed by infusion of GLP-1 agonists or vehicle (artificial CSF) into the right lateral ventricle for 14 days. Choline acetyltransferase (ChAT)–positive immunoreactivity as the marker for cholinergic cell bodies within the basal forebrain, showed that GLP-1R stimulation significantly decreased the loss of ChAT-positive cell bodies in the lesion area compared to the vehicle-treated lesion group,[107] to an extent that there was no difference between rats with a lesion that

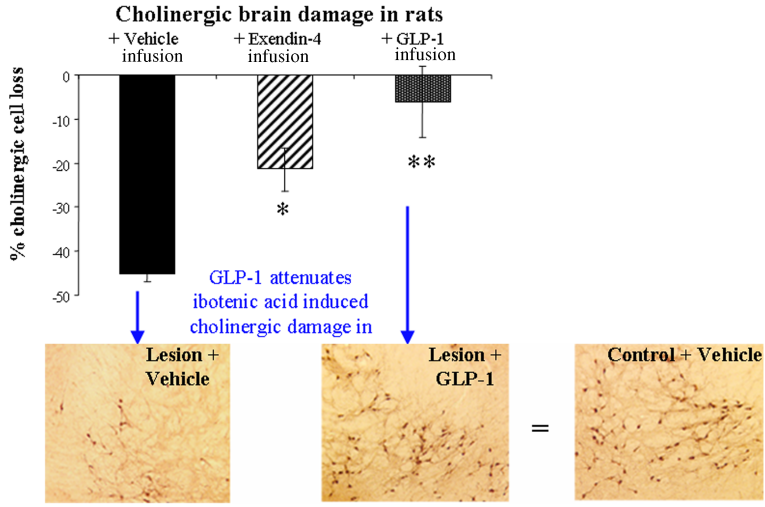

FIGURE 13. Percent difference in the Abercrombie-corrected number of ChAT-immunoreactive cell bodies in the lesioned basal nucleus relative to the intact contralateral basal nucleus in sham and ibotenic acid animals receiving i.c.v. infusion of vehicle, GLP-1 (0.8 nM/kg/min) or its long-acting analogue, exendin-4 (0.08 nM/kg/min), for 14 days. Vertical error bars represent the standard error of the difference between the means. Significant difference from ibotenic acid vehicle group: $*P<.05$ and $**P<.01$. Representative areas of ChAT-positive stained cells in the nucleus basalis are shown for control + vehicle (i.e., no lesion) and animals with a lesion that were either administered vehicle or GLP-1. There is no discernible difference between animals without a lesion and those with +GLP-1, in relation to ChAT-positive neurons.[107]

were administered GLP-1 and control rats without a lesion administered vehicle (FIG. 13).

Based on our combined studies, we hypothesize that GLP-1 and analogues possess neurotrophic capabilities that are not specific for excitotoxic or, indeed, cholinergic neuronal death, but may represent a trophic property for all neuronal GLP-1R expressing cells.[95] Ongoing studies are currently testing this hypothesis in a wide variety of degenerative models of clinical relevance. This is particularly interesting as the receptors for NGF and related neurotrophins (tyrosine kinase family members) and for GLP-1 (G protein–coupled receptor family member) have been characterized and are vastly different. Furthermore, there is minimal structural similarity between GLP-1 (30 amino acids) and the far larger NGF (118 amino acids). Nevertheless, there are numerous cross-overs in the biochemical pathways that are initiated by activation of their respective receptors that lead towards cell survival. This suggests that GLP-1R activation may prove a useful therapeutic strategy in neurodegenerative disorders, particularly as a loss of the TrkA receptor for NGF, together with the large size of the protein, may negatively impact its value in some disorders, such as AD. In contrast, the brain uptake of GLP-1 is surprisingly high.[116]

The abundance and localization of the GLP-1R in brain and the peripheral nervous system in health, aging, and disease remain to be fully elucidated, but have not been reported to be lost.

SUMMARY

Many advances have been made during the last decade to progress our understanding of the key mechanisms that regulate cell survival and death in response to a wide number of physiological insults, via programmed cell death. A critical process in sculpting the developing brain, the very same biochemical cascades, albeit activated by different stimuli, appear to be consistently involved in a wide variety of neurodegenerative disorders. Certainly programmed cell death is not the sole mediator of cell loss seen in these disorders but, as assessed with a number of experimental mouse models, it represents a key mechanism. As illustrated in FIGURES 1 and 11, the molecular complexity of the cascades associated with programmed cell death or survival offers numerous potential targets for modulation. Few, however, are classical or validated, which has forced groups associated with drug design to think "outside the box" and collaborate more broadly with experts in molecular and cellular biology to characterize un-validated targets. We have reviewed three novel and different therapeutic strategies with potential for a wide variety of neurodegenerative disorders and described small compounds and peptides that already possess interesting pharmacological profiles in cellular and *in vivo* models. It is important to define the molecular point-of-no-return associated with specific acute (e.g., stroke and head trauma) and chronic (e.g., AD, PD, and ALS) disorders, to determine the point when neurons become irreversibly committed to die, and the window of therapeutic opportunity that this provides. In addition, it remains to be determined whether or not the toxicological profile associated with such strategies will allow their use for intervention in humans. It could well be that a synergistic combination of rational therapeutic approaches may prove optimal and, for chronic disorders, likely will require the initiation of intervention well before a cellular "suicide" decision has been made. This could potentially slow or halt processes that gradually render neurons dysfunctional well before their loss of function leaves them all but worthless.

ACKNOWLEDGMENTS

This paper is dedicated to our collaborator and close friend, Arnold Brossi, University of North Carolina (Chapel Hill, NC), on the occasion of his 81st birthday. We are grateful to Harold W. Holloway, Laboratory of Neurosciences, Intramural Research Program, National Institute on Aging, National Institutes of Health, for his critical input into this article.

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