Novel nitrates as NO mimetics directed at Alzheimer’s disease

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Abstract. GT 1061 is a novel therapeutic agent that is in Phase I clinical studies for Alzheimer’s disease. GT 1061 is one of a family of novel nitrates that have demonstrated neuroprotective properties and cognition- and memory-enhancing properties in animal models. The prototype of this family, GT 715, has been reported effectively to dissociate the neuromodulatory and the systemic hypotensive effects of nitrates, the latter seriously limiting the therapeutic use of classical nitrates. Further data on the novel nitrates, GT 715 and GT 061, are presented in (a) the malonate-lesion rat model of excitotoxic neurodegeneration, and (b) the reversal of a scopolamine-induced cognition deficit in the Morris water task which tests spatial memory. These data exemplify and reinforce the combined neuroprotective and cognition enhancing properties observed in this family of NO mimetic therapeutic agents. NO mimetics, that mimic the biological activity of NO, will bypass cholinergic receptor activation and are anticipated to provide multiple pathways of treating and circumventing dementia. NO mimetic activation of soluble guanylyl cyclase and cGMP formation in the brain represents one element of an effective neuroprotective strategy. Substantial evidence suggests that NO mimetics may display cGMP-dependent and cGMP-independent activity and may operate via multiple biochemical signaling pathways, both to ensure the survival of neurons subjected to stress and also to provide cognition-enabling pathways to circumvent dementia, providing a combined neuroprotective and cognition-enabling approach to anti-neurodegenerative therapy.

Keywords: Cognition, dementia, nitric oxide, cGMP, nitrate, Alzheimer’s, neurodegeneration

1. Introduction

GT 1061 is a novel therapeutic agent, initially targeted at treatment of mild to moderate Alzheimer’s disease (AD), that has received regulatory approval for a phase Ia study in healthy aged volunteers. GT 1061 is one of a family of compounds, coined S-nitrates, that have demonstrated neuroprotective as well as cognition- and memory-enhancing properties in animal models. All members of this class tested to date in behavioural models of dementia show cognition-enhancing activity as reported for the S-nitrate, GT 715 [1], and various members manifest additional biological activity, including anticonvulsant and analgesic activity, and AMPA receptor modulation [2]. Neuroprotective properties have been shown (a) in the middle cerebral artery occlusion (MCAO) rat model of focal ischemic stroke [3], (b) in the 6-hydroxydopamine-lesion rat model of Parkinson’s disease, and (c) in the malonate-lesion rat model of excitotoxic neurodegeneration (vide infra).

S-Nitrates have been studied in a number of behavioral tests used to demonstrate memory improvement and the reversal of cognition deficits in rat models of dementia, employing a variety of experimental approaches, including: (a) injection of the muscarinic receptor antagonist scopolamine [1]; (b) administration of the cholinergic neurotoxin 192 IgG-saporin via in-
Table 1

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Test</th>
<th>Control</th>
<th>Drug admin.</th>
<th>Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scopolamine i.p.</td>
<td>Morris water maze (MWT)- fixed platform</td>
<td>tacrine&lt;sup&gt;a&lt;/sup&gt;</td>
<td>s.c.</td>
<td>young</td>
</tr>
<tr>
<td>IgG-saporin i.c.v.</td>
<td>MWT – fixed and moving platform</td>
<td>donepezil&lt;sup&gt;b&lt;/sup&gt;</td>
<td>i.p. and i.p.</td>
<td>young</td>
</tr>
<tr>
<td>Scopolamine i.p.</td>
<td>Step through passive avoidance test (STPA)</td>
<td>n/a</td>
<td>p.o.</td>
<td>young</td>
</tr>
<tr>
<td>IgG-saporin i.c.v.</td>
<td>Delayed visual matching to sample (VMTS)</td>
<td>donepezil</td>
<td>p.o. and i.p.</td>
<td>young</td>
</tr>
<tr>
<td>IgG-saporin i.c.v.</td>
<td>Contextual memory after STPA training</td>
<td>donepezil</td>
<td>p.o.</td>
<td>old/young</td>
</tr>
<tr>
<td>Aβ1-40 i.c.v.</td>
<td>MWT – fixed platform</td>
<td>donepezil</td>
<td>p.o.</td>
<td>young</td>
</tr>
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<sup>a</sup>Cognex.  
<sup>b</sup>Aricept.

tracerebroventricular infusion and other routes [4]; and (c), chronic, daily, bilateral, intracerebroventricular infusion of amyloid-β peptide (Aβ1–40) [5]. In addition to these models of brain injury, beneficial effects of S-nitrates have been demonstrated in normal aging, in that GT 715 decreases the intrusion of high amplitude, low frequency spindle activity in the cerebrocortical electroencephalograph (EEG) during periods of waking immobility in aged rats. This effect on neurophysiological function is associated with improved attention, and therefore performance, during learning tasks. In these assays acetylcholinesterase inhibitor drugs, the mainstay of AD pharmacotherapy, were used for comparison and to validate models. Studies completed for GT 1061 are summarized in Table 1.

In this paper, we provide further data on neuroprotective and cognition-enhancing properties of S-nitrates and briefly summarize the design rationale and potential of this new family of therapeutic agents.

2. Materials and methods

The experiments described in this study were carried out in accordance with the guidelines established by the Canadian Council on Animal Care, and were approved by the Queen’s University Animal Care Committee.

2.1. Malonate-lesion model

Adult male, Sprague-Dawley rats were anesthetized with sodium pentobarbital, and a stereotaxic injection of 1.5 µl saline containing 2 µmol malonate was made into the right striatum. For drug administration, GT 715 or vehicle (DMSO) were given by s.c. injection every hour for 6 hours, with the first dose given 2 hours after the malonate injection. Each dose of GT 015 was 200 µmol/kg. At 72 hours after malonate injection, apomorphine (1 mg/kg) was given by subcutaneous injection, and ipsilateral turning behaviour was determined at 15 minute intervals for 60 minutes. Striatal tissue was collected 96 hours after malonate injection for determination of γ-aminobutyric acid (GABA) content by HPLC coupled with precolumn derivatization with o-phthaldehyde and fluorescence detection. GABA content on the ipsilateral (injected) side was expressed as a percentage of the contralateral (control) side. Statistical comparisons were made using Student’s t-test for unpaired data.

2.2. Scopolamine-impaired acquisition of a spatial learning task

Adult, male Long-Evans rats (250–300 g) were used to test spatial learning in the Morris water maze as previously detailed [1,6]. Rats were given a total of 12 trials, grouped in 3 trial blocks (i.e., 4 trials/block; approximately 5 min between blocks). The latency to locate the hidden platform was measured by an experimenter standing in a fixed position relative to the pool. Different groups of rats received the following drug treatments: scopolamine (0.5 mg/kg, i.p.) + DMSO vehicle (1 ml/kg, s.c.), scopolamine + GT 061 (0.1–10 mg/kg, s.c.) in DMSO, saline + saline (1 ml/kg). Scopolamine was always administered 25 min before testing, and drug or vehicle injections were given 5 min later (20 min before testing). All drug administrations were given in a volume of 1 ml/kg body weight.

3. Results

Malonate is a reversible inhibitor of the mitochondrial (complex II) enzyme, succinate dehydrogenase. Malonate injections into the brain of experimental animals produce energy depletion, secondary excitotoxicity, and free radical production that ultimately leads to neuronal cell death [7]. This model of neuronal injury has been used extensively in testing for potential new neuroprotective agents, since a defect in en-
ergy metabolism that leads to slow excitotoxic neuronal death is considered to be a potential mechanism of the neuronal injury that characterizes many neurodegenerative diseases [8]. GT 715 significantly decreased the brain injury induced by intrastriatal injections of the mitochondrial toxin, malonate. This neuroprotective effect was manifested at both the behavioural and neurochemical levels (Fig. 1). Preservation of GABA content in the striatum after malonate injection suggests that GABAergic neurons in this structure have been rescued from cell death. GABA is the primary neurotransmitter for neurons in the neostriatum, and therefore GABA content can be used as an index of the neuronal cell population in this brain region. Decreased response to apomorphine also indicates that neuronal injury induced by malonate was significantly inhibited by GT 715 administration, and that normal function within the neostriatum was maintained.

In AD, loss of cholinergic neurons and subsequent deficits in cholinergic neurotransmission in the hippocampus and cerebral cortex, are strongly correlated with clinical signs of cognitive impairment and dementia. Central cholinergic muscarinic receptor blockade produces profound cognitive impairments in human and animal subjects, thus the use of cholinergic muscarinic antagonists, such as scopolamine, in animal models to mimic the cognitive impairment observed in AD is well established, and has proven to be a useful model system for understanding and developing treatment strategies for neurodegenerative diseases in humans [9]. GT 1061 is a salt form of GT 061. GT 061 was seen to reverse the cognitive impairment induced by scopolamine in the Morris water task (Fig. 2). Animals that received scopolamine plus vehicle failed to acquire the task over 3 trial blocks, whereas, in contrast, animals that received scopolamine plus GT 061 (10 mg/kg) did progressively better over the 3 trial blocks, such that by the second and third trial block they performed at a level significantly better than vehicle treated animals.

4. Discussion

4.1. NO and neuroprotection

NO signaling is essential for normal physiological function in the CNS, including learning and memory, and is compromised in many disease states including neurodegenerative disorders, where reduced intracellular NO levels may result from upstream blockade, as in cases where cholinergic neurons are damaged and acetylcholine is depleted. NO can serve as a retrograde synaptic messenger, as an intracellular messenger, and as a lateral diffusible messenger in the CNS. NO plays a critical role in signal transduction cascades that are compromised in AD and thereby contribute to the symptoms of cognitive impairment and dementia that characterize AD. NO activates soluble guanylyl cyclase (sGC) to release cyclic guanosine-2′:3′-monophosphate (cGMP); NO/cGMP signaling is important for modulating synaptic transmission and plasticity in brain regions, such as the hippocampus and cerebral cortex, which are critical for learning and memory [10, 11]. There is evidence that the effects of NO/cGMP in memory formation and retrieval are mediated by both protein kinase G (PKG)-dependent and independent pathways, and moreover, that NO may positively impact learning, memory and cognition through cGMP independent pathways [12]. Compounds that mimic...
the effects of NO, which one might term NO mimetics, will bypass cholinergic receptor activation and are anticipated to provide multiple pathways of treating and circumventing dementia.

Earlier theories of glutamate-induced excitotoxic neurodegeneration pushed a central role for the elevation of postsynaptic NO levels as causative in neuronal damage primarily via generation of free radicals and the oxidizing cytotoxin, peroxynitrite [13]. One of the initiating events in excitotoxic, neuronal cell death is excessive release of the excitatory amino acid glutamate. Prolonged or cytotoxic activation of the N-methyl-D-aspartate (NMDA) subtype of ionotropic glutamate receptors has long been associated with ischemic brain injury [14]. Prolonged NMDA receptor activation allows the excessive influx of calcium into the postsynaptic neuron, which initiates multiple processes that contribute to cellular injury and death, including the activation of proteases, and inhibition of mitochondrial respiration leading to failure of cellular energy stores and activation of cell death programs. The increase in intracellular calcium also results in activation of a number of calcium/calmodulin-dependent enzymes, including constitutive nitric oxide synthase (NOS). Excessive production of NO, via excitotoxic activation of NMDA receptors, may lead to generation of cytotoxic peroxynitrite, which would be a contributing factor in ischemic injury and cell death as a consequence of inhibition of mitochondrial energy production [15,16]. Studies that have supported a role for NO as a neurodestructive agent include observations of neuroprotection in NOS knockout mice and on treatment with NOS inhibitors [17–20]. However, several studies also clearly demonstrate the anti-neurodegenerative properties of NO and of NO/cGMP signaling [21–23]. The hypothesis of induction or activation of NOS as a central causal factor in neuronal damage cannot be universal. Modifications of this paradigm that posit a threshold NO concentration above which neurotoxicity is observed, or that suggest neuroprotective roles for nNOS and neurodestructive roles for iNOS, are likely also to prove too simplistic.

Lipton’s seminal work on the interaction of NO with NMDA receptors showed that some nitrovasodilators are neuroprotective in models of NMDA receptor-mediated excitotoxic neuronal injury, and provided a role for NO as a neuroprotective agent in inhibiting NMDA receptor-mediated excitotoxicity [16]. The putative mechanism of regulation of NMDA receptor activity by endogenous NO and exogenous NO donors involves conformational changes to the NMDA receptor induced by reversible protein thiol S-nitrosation [24], providing a cGMP-independent neuroprotection pathway for NO [16].

The biological actions of NO can be classified as either cGMP dependent or independent, and amongst the cGMP independent properties are protein nitrosation, protein nitration, and antioxidant action. An NO donor was shown to be neuroprotective against an oxidative stress-induced neuronal cell injury in the substantia nigra subjected to oxidative stress [25]. There is good evidence that NO can act as a potent chain-breaking antioxidant and that certain organic nitrates may manifest antioxidant activity [26,27]. Thus, the neuroprotective effects of NO and NO mimetics may result from the action of NO as an antioxidant; via NO-mediated inhibition of caspases; via NO-mediated modulation of NMDA receptor activity; or via cGMP-dependent pathways, such as those that inhibit apoptosis [28–30]. Therefore, sGC activation and cGMP formation in the brain represents one element of an effective NO mimetic neuroprotective strategy.

Fig. 2. GT 061 induced reversal of scopolamine-impaired task acquisition in the Morris water test. (a) Escape latency expressed as mean ± s.e.m., n = 7, where * represents p < 0.05 by t-test: vehicle (○); GT 061 (10 mg/kg) (▲). (b) Dose dependence for GT 061 treatment group in trial block 3 expressed as the mean ± s.e.m. of the average scores, n = 7, where * represents p < 0.05 by two-way ANOVA using Tukey post test.
4.2. NO mimetics as anti-neurodegenerative agents: Neuroprotection + cognition enhancement

The NO/cGMP signal transduction system is linked to several potential neuroprotective signaling pathways in the brain. NO possesses neuroprotective properties related to activation of sGC and the production of cGMP, since cGMP has been found to protect neurons against excitotoxic injury [31], and to promote neuronal survival and inhibit apoptotic cell death in a number of neuronal cell types [22]. Furthermore, cyclic nucleotides (cGMP and cAMP) attenuate lipid peroxidation-mediated neuronal injury [32], and cGMP decreases both resting intracellular Ca\(^{2+}\) levels and the elevations in intracellular Ca\(^{2+}\) concentrations that follow exposure to glutamate [21]. It has also been reported that elevating cellular levels of cGMP depresses excitatory synaptic transmission in the hippocampus, possibly via a direct, PKG-independent interaction between cGMP and the \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) subtype of excitatory amino acid receptors [33]. Soluble \(\beta\)-amyloid reservoir protein (APP) has neuroprotective properties that have been attributed to selective elevation of intracellular cGMP levels and activation of PKG [34]. Conversely, elevation of cGMP leads to inhibition of the proinflammatory action of amyloid-\(\beta\) peptide itself, on microglia [35]. Microglial activation leading to release of proinflammatory cytokines and neurotoxic factors is strongly implicated in the pathogenesis of neurodegenerative disorders [36]. In microglial cell culture, inhibitors of cytokine release, including NO donors and cGMP analogs, operate via cGMP/PKG signaling and the mitogen-activated protein kinase (MAPK) cascade (see Fig. 3 and caption for all further abbreviations) [37].

Studies on neuroprotection resulting from preconditioning also emphasize a key role for NO and the importance of MAPK signaling cascades. In cell culture experiments, operation of NO/cGMP/PKG pathways, resulting from nNOS activation, mediated activation of signaling cascades via ERK1/2 (extracellular signal-regulated kinases) and c-Jun, leading to up-regulation and activation of proteins including BDNF, thioredoxin and superoxide dismutase, as well as the anti-apoptotic factor, Bcl-2 [38,39]. In this work, a role for protein S-nitrosation was ruled out. In other work, neuronal preconditioning was reported to be mediated by NOS activation, and replicated by NO donors, via NMDA-dependent, Ras-dependent, but cGMP-independent pathways [40]; but again ERK1/2 phosphorylation/activation was observed to be essential for neuroprotection.

The discovery that the ERK cascade mediates hippocampus-dependent long term memory (LTM) and amygdala/hippocampus-dependent fear condition-
ing was made only in 1998, but has led to considerable research demonstrating the importance of this signal cascade in several brain regions [41–45]. ERK1/2 are members of the MAPK super-family (see Fig. 3 caption for abbreviations). ERK was shown to be activated in the rat hippocampal CA1 region following NMDA receptor stimulation [46], but has now been shown to be activated by a number of stimuli in the hippocampus, cortex, and amygdala [47–50]. The membrane-associated G-protein, Ras can activate the ERK pathway via the kinase Raf-1, which is a MAPKKK (MEK kinase) (Fig. 3) [51]. Stimulation of several neurotransceptors, including NMDA, serotonin, muscarinic and nicotinic acetylcholine receptors can lead to ERK activation via the protein kinases, PKA or PKC. CREB can be activated and phosphorylated via CaMK IV, PKA, and RSK2, the last mediating the activation of CREB by ERK [52].

CREB activation, which can be elicited by NO [10, 53,54], is a focus of investigations into the cellular molecular mechanisms underlying both depression and cognition [41,44,55–60], but understanding of the detailed upstream pathways is incomplete, owing to the number and complexity of signaling cascades and substantial cross-talk (Fig. 3). CaMK IV may drive fast-onset CREB-activation because of its constitutive nuclear expression and its responsiveness to activation by fast-moving Ca\(^{2+}\) waves generated at distant synapses [61]. The PKA and ERK pathways may activate CREB in a slower manner. The combination of multiple signaling pathways may be advantageous for the precise control of gene expression and the integration of multiple converging signals for optimal activation of CREB [61,62].

NO/cGMP signaling is intimately linked with behavioural responses, learning, and memory [63–68]. Animal behavioural studies have shown that NO is involved in both short and long term learning and memory [54,69,70]. Long term potentiation (LTP), centrally important to learning and memory, has early and late phases that require NO/cGMP signaling and CREB phosphorylation. Recent results suggest that cGMP-dependent protein kinase (PKG) which is activated by NO/cGMP, provides a parallel pathway to PKA-signaling in both phases of LTP, with PKG and PKA pathways performing complementary roles [10]. NO/cGMP and PKG contribute to CREB phosphorylation, in part mediated by the ERK cascade, but probably also mediated in part via CaMK [10]. Interestingly, experiments with YC-1 an agent that augments sGC activation, produced an enhancement of LTP in rat hippocampus and amygdala via an NO/cGMP/PKG/ERK pathway culminating in CREB phosphorylation [71].

One factor that differentiates NO from other messenger molecules is transmembrane diffusion, allowing retrograde signal transduction stimulating cGMP in the presynaptic terminus, but also potentially sensitizing adjacent postsynaptic neurons as a lateral diffusable messenger. The exact role of cGMP signaling in learning, memory, and affective behaviour is not fully understood. However, there are a number of important ideas that are emerging: the importance of linking presynaptic and postsynaptic activity in a pathway specific manner; the observation that cGMP signaling in LTP may be brief and phasic; and the importance of cGMP signaling in the consolidation phase of learning and memory (i.e. in the 1–2 h after new information is received, before it is encoded in a long-term memory store). Clearly, NO mimetics that manifest cGMP-dependent and independent activities may operate via multiple biochemical pathways, to ensure the survival of neurons subjected to ischemic injury, Ca\(^{2+}\) overload, or oxidative stress, and also to provide cognition-enabling pathways to circumvent dementia. Emerging research would support activation of ERK1/2 and CREB as important in both neuroprotective and cognition-enhancing pathways.

4.3. Nitrates as NO mimetic therapeutics in neurodegeneration

GTN is the archetypal classical nitrate, yet in some ways GTN is atypical, since GTN is a much more potent hypotensive agent than most other classical nitrate vasodilators and the clinical phenomenon of nitrate tolerance is more profound and complex for GTN [73–75]. As a drug, GTN is the product of serendipity, a NO mimetic with cardiovascular and venodilator selectivity, and with a clinical safety record proven over 130 years. However, GTN is contraindicated for CNS indications, such as cerebral ischemia, because of peripheral hypotension and tolerance [76,77]. GTN and other nitrates are often described as NO donors, requiring biotransformation (or bioactivation) to NO, but no protein capable of bioactivation of GTN to NO is known [78], and at pharmacological concentrations, NO release in target tissues can be at a level too low to measure [79].

The biological and medicinal chemistry of nitrates has recently been thoroughly reviewed [74]. The biological activity of nitrates is NO mimetic, and one might hypothesize that nitrates are able to exploit bioactivation pathways for selectivity, but in contrast to NO
donors such as nitrosothiols, nitrates, at therapeutic doses, will not release large, potentially harmful fluxes of NO. Thus, with over a century of human clinical experience, nitrates represent ideal NO mimetic therapeutics. Recent data on hybrid nitrates, comprising a classical nitrate grafted onto a primary pharmacophore, have yielded exciting data, although in most cases it is difficult to dissect and attribute the activity to the pharmacophore versus the nitrate moiety. Two recent, positive animal studies on hybrid nitrates, of interest to AD and neurodegeneration, have reported both microglial activation and amyloid clearance [80,81], and inhibition of microglial activation [82]. Several hybrid nitrate therapeutics, in particular the so called NO-NSAIDs (NO donor non-steroidal anti-inflammatory drugs), have received regulatory approval and completed human clinical trials [83].

The putative bioactivation of nitrates by conversion to NO is a 3e− reduction which requires oxygen atom transfer, whereas the conversion to the thiol nitrosating agent NO+ is a 2e− reduction; both conversions would lead to the depletion of reducing equivalents which under pathophysiological conditions, such as in oxidative stress, would be detrimental. The incorporation of an additional functionality into nitrates which would assist bioactivation and attenuate reductant depletion represents a strategy for design of novel nitrate NO mimetics. Of course, this strategy is also amenable to development of hybrid nitrates. The S-nitrate, GT 715, represents a prototype for one such approach [84,85]. GTN and GT 715 were shown to exert differential effects on cardiovascular function, with GT 715, being a weaker vasodilator with minimal effects on mean arterial pressure in the whole animal compared to GTN [77].

GT 715 was both more potent and more effective as an activator of sGC in the brain, and more effective in elevating cGMP levels in hippocampal brain slices, compared to GTN, whereas GTN produced a much greater accumulation of cGMP in vascular tissue [1]. Thus, the neuromodulatory and systemic hypotensive effects of nitrates can clearly be dissociated; furthermore, since the nitrate functional group is inherently lipophilic, nitrates may be developed with good CNS bioavailability.

The neuroprotective effects of GT 715 in the malonate stroke model of excitotoxic neurodegeneration (Fig. 1) and the MCAO model of ischemic stroke [77] demonstrate the neuroprotective properties of S-nitrates. The reversal of cognitive deficits in animal models of dementia by S-nitrates, exemplified by the effects of GT 715 [1] and of GT 061 (Fig. 2) in behavioural tests, demonstrate the cognition enhancing properties of these drugs. GT 1061 is a pharmaceutically appropriate salt form of GT 061 that was selected for clinical trials on the basis of factors including efficacy in a range of animal models (Table 1) and demonstrated anticonvulsant activity, which are the focus of manuscripts in preparation. The combination of neuroprotection with cognition enhancement presents exciting potential for new approaches to anti-neurodegenerative therapy.

**Acknowledgements**

G Btherapeutics Ltd (Canada) for financial support.

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