Abstract: The present invention relates to methods and compositions for treating neuropathic pain and neuropsychiatric disorders by administering agents that are effective in reducing the effective amount, inactivating, and/or inhibiting the activity of a Na⁺-K⁺-2Cl⁻ (NKCC) cotransporter. In certain embodiments, the Na⁺-K⁺-2Cl⁻ co-transporter is NKCC1.
For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.
METHODS AND COMPOSITIONS FOR THE TREATMENT OF NEUROPSYCHIATRIC AND ADDICTIVE DISORDERS

Reference to Related Applications

This application claims the benefit of U.S. Patent Application No. 11/549,274, filed October 13, 2006; U.S. Patent Application No. 60/727,564, filed October 17, 2005; U.S. Patent Application No. 60/727,318, filed October 17, 2005; and U.S. Patent Application No. 11/251,724, filed October 17, 2005, the disclosures of which are incorporated herein by reference in their entireties.

Technical Field of the Invention

The present invention relates to methods and compositions for treating selected conditions of the central and peripheral nervous systems employing non-synaptic mechanisms. More specifically, the present invention relates to methods and compositions for treating neuropsychiatric and addictive disorders by administering agents that modulate expression and/or activity of sodium-potassium-chloride co-transporters.

Background of the Invention

Neuropathic pain and nociceptive pain differ in their etiology, pathophysiology, diagnosis and treatment. Nociceptive pain occurs in response to the activation of a specific subset of peripheral sensory neurons, the nociceptors. It is generally acute (with the exception of arthritic pain), self-limiting and serves a protective biological function by acting as a warning of on-going tissue damage. It is typically well localized and often has an aching or throbbing quality. Examples of nociceptive pain include post-operative pain, sprains, bone fractures, burns, bumps, bruises, inflammation (from an infection or arthritic disorder), obstructions and myofascial pain. Nociceptive pain can usually be treated with opioids and non-steroidal anti-inflammatory drugs (NSAIDS).

Neuropathic pain is a common type of chronic, non-malignant, pain, which is the result of an injury or malfunction in the peripheral or central nervous system and serves no protective biological function. It is estimated to affect more than 1.6 million people in the U.S. population. Neuropathic pain has many different etiologies, and may occur, for example, due to trauma, diabetes, infection with herpes zoster (shingles), HIV/AIDS...
peripheral neuropathies, late-stage cancer, amputation (including mastectomy), carpal tunnel syndrome, chronic alcohol use, exposure to radiation, and as an unintended side-effect of neurotoxic treatment agents, such as certain anti-HIV and chemotherapeutic drugs.

In contrast to nociceptive pain, neuropathic pain is frequently described as "burning", "electric", "tingling" or "shooting" in nature. It is often characterized by chronic alldynia (defined as pain resulting from a stimulus that does not ordinarily elicit a painful response, such as light touch) and hyperalgesia (defined as an increased sensitivity to a normally painful stimulus), and may persist for months or years beyond the apparent healing of any damaged tissues.

Neuropathic pain is difficult to treat. Analgesic drugs that are effective against normal pain (e.g., opioid narcotics and non-steroidal anti-inflammatory drugs) are rarely effective against neuropathic pain. Similarly, drugs that have activity in neuropathic pain are not usually effective against nociceptive pain. The standard drugs that have been used to treat neuropathic pain appear to often act selectively to relieve certain symptoms but not others in a given patient (for example, relief of alldynia, but not hyperalgesia). For this reason, it has been suggested that successful therapy may require the use of multiple different combinations of drugs and individualized therapy (see, for example, Bennett, Hosp. Pract. (Off Ed). 33:95-98, 1998). Treatment agents typically employed in the management of neuropathic pain include tricylic antidepressants (for example, amitriptyline, imipramine, desipramine and clomipramine), systemic local anesthetics, and anti-convulsants (such as phenytoin, carbamazepine, valproic acid, clonazepam and gabapentin).

Many anti-convulsants originally developed for the treatment of epilepsy and other seizure disorders have found application in the treatment of non-epileptic conditions, including neuropathic pain, mood disorders (such as bipolar affective disorder), and schizophrenia (for a review of the use of anti-epileptic drugs in the treatment of non-epileptic conditions, see Rogawski and Loscher, Nat. Medicine, 10:685-692, 2004). It has thus been suggested that epilepsy, neuropathic pain and affective disorders have a common pathophysiological mechanism (Rogawski & Loscher, ibid; Ruscheweyh & Sandkuhler, Pain 105:327-338, 2003), namely a pathological increase in neuronal excitability, with a corresponding inappropriately high frequency of spontaneous firing of neurons. However, only some, and not all, antiepileptic drugs are effective in treating neuropathic pain, and furthermore such antiepileptic drugs are only effective in

Epilepsy is characterized by abnormal discharges of cerebral neurons and is typically manifested as various types of seizures. Epileptiform activity is identified with spontaneously occurring synchronized discharges of neuronal populations that can be measured using electrophysiological techniques. This synchronized activity, which distinguishes epileptiform from non-epileptiform activity, is referred to as "hypersynchronization" because it describes the state in which individual neurons become increasingly likely to discharge in a time-locked manner with one another. Hypersynchronized activity is typically induced in experimental models of epilepsy by either increasing excitatory or decreasing inhibitory synaptic currents, and it was therefore assumed that hyperexcitability per se was the defining feature involved in the generation and maintenance of epileptiform activity. Similarly, neuropathic pain was believed to involve conversion of neurons involved in pain transmission from a state of normal sensitivity to one of hypersensitivity (Costigan & Woolf, *J. Pain* 1:35-44, 2000). The focus on developing treatments for both epilepsy and neuropathic pain has thus been on suppressing neuronal hyperexcitability by either: (a) suppressing action potential generation; (b) increasing inhibitory synaptic transmission; or (c) decreasing excitatory synaptic transmission. However, it has been shown that hypersynchronous epileptiform activity can be dissociated from hyperexcitability and that the cation-chloride cotransport inhibitor furosemide reversibly blocked synchronized discharges without reducing hyperexcited synaptic responses (Hochman et al. *Science* 270:99-102, 1995).


Neuropsychiatric disorders, including anxiety disorders, are generally treated by counseling and/or with drugs. Many of the drugs currently employed in the treatment of such disorders have significant negative side effects, such as tendencies to induce dependence.

The cation-chloride co-transporters (CCCs) are important regulators of neuronal chloride concentration that are believed to influence cell-to-cell communication, and various aspects of neuronal development, plasticity and trauma. The CCC gene family consists of three broad groups: Na+-Cl⁻ co-transporters (NCCs), K+-Cl⁻ co-transporters
(KCCs) and Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) co-transporters (NKCCs). Two NKCC isoforms have been identified: NKCC1 is found in a wide variety of secretory epithelia and non-epithelial cells, whereas NKCC2 is principally expressed in the kidney. For a review of NKCC structure, function and regulation see, Haas and Forbush, *Annu. Rev. Physiol.* 62:515-534, 2000. Randall et al. have identified two splice variants of the Slc12a2 gene that encodes NKCCI, referred to as NKCCIa and NKCCIb (Am. J. Physiol. 273 (Cell Physiol. 42):C1267-1277, 1997). The NKCCI a gene has 27 exons, while the splice variant NKCCIb lacks exon 21. The NKCCIb splice variant is expressed primarily in the brain. NKCCIb is believed to be more than 10% more active than NKCCIa, although it is proportionally present in a much smaller amount in the brain than is NKCCIa. It has been suggested that differential splicing of the NKCCI transcript may play a regulatory role in human tissues (Vibat et al. *Anal. Biochem.* 298:218-230, 2001). Na-K-Cl co-transport in all cell and tissues is inhibited by loop diuretics, including furosemide, bumetanide and benzmetanide.

Na-K-2Cl co-transporter knock-out mice have been shown to have impaired nociception phenotypes as well as abnormal gait and locomotion (Sung et al. *JnI. Neurosci.* 20:7531-7538, 2000). Delpierre and Mount have suggested that NKCCI may be involved in pain perception (Ann. Rev. Physiol. 64:803-843, 2002). Laird et al. recently described studies demonstrating reduced stroking hyperalgesia in NKCCI knock-out mice compared to wild-type and heterozygous mice (Neurosci. Letts. 361:200-203, 2004). However, in this acute pain model no difference in punctuate hyperalgesia was observed between the three groups of mice. Morales-Aza et al. have suggested that, in arthritis, altered expression of NKCCI and the K-Cl co-transporter KCC2 may contribute to the control of spinal cord excitability and may thus represent therapeutic targets for the treatment of inflammatory pain (Neurobiol. Dis. 17:62-69, 2004). Granados-Soto et al. have described studies in rats in which formalin-induced nociception was reduced by administration of the NKCC inhibitors bumetanide, furosemide or piretanide (Pain 114:231-238, 2005). While the formalin-induced acute pain model is extensively used, it is believed to have little relevance to chronic pain conditions (Walker et al. Mol. Med. Today 5:319-321, 1999). Co-treatment of brain damage induced by episodic alcohol exposure with an NMDA receptor antagonist, non-NMDA receptor and Ca\(^{2+}\) channel antagonists together with furosemide has been shown to reduce alcohol-dependent cerebrocortical damage by 75-85%, while preventing brain hydration and electrolyte elevations (Collins et al, *FASEB J*, 12:221-230, 1998). The authors stated that the results
suggest that furosemide and related agents might be useful as neuroprotective agents in alcohol abuse. Willis et al. have published studies indicating that nedocromil sodium, furosemide and bumetanide inhibit sensory nerve activation to reduce the itch and flare responses induced by histamine in human skin in vivo. Espinosa et al. and Ahmad et al. have previously suggested that furosemide might be useful in the treatment of certain types of epilepsy (Medicina Espanola 61:280-281, 1969; and Brit. J. Clin. Pharmacol. 3:621-625, 1976).

As with epilepsy, the focus of pharmacological intervention in neuropathic pain has been on reducing neuronal hyperexcitability. Most agents currently used to treat neuropathic pain target synaptic activity in excitatory pathways by, for example, modulating the release or activity of excitatory neurotransmitters, potentiating inhibitory pathways, blocking ion channels involved in impulse generation, and/or acting as membrane stabilizers. Conventional agents and therapeutic approaches for the treatment of neuropathic pain and neuropsychiatric disorders thus reduce neuronal excitability and inhibit synaptic firing. One serious drawback of these therapies is that they are nonselective and exert their actions on both normal and abnormal neuronal populations. This leads to negative and unintended side effects, which may affect normal CNS functions, such as cognition, learning and memory, and produce adverse physiological and psychological effects in the treated patient. Common side effects include oversedation, dizziness, loss of memory and liver damage. There is therefore a continuing need for methods and compositions for treating neuronal disorders that disrupt hypersynchronized neuronal activity without diminishing the neuronal excitability and spontaneous synchronization required for normal functioning of the peripheral and central nervous systems.

Addictive disorders, such as eating disorders (including obesity), addiction to narcotics, sexual addiction, alcoholism and smoking, are a major public health problem that impacts society on multiple levels. It has been estimated that substance abuse costs the US more than $484 billion per year. Current strategies for the treatment of addictive disorders include psychological counseling and support, use of therapeutic agents or a combination of both. A variety of agents known to affect the central nervous system have been used in various contexts to treat a number of indications related directly or indirectly to addictive behaviors. For example, the combination of phentermine and fenfluramine was used for many years to exert an anorectic effect to treat obesity.
Topiramate is an anti-convulsant that was originally developed as an anti-diabetic agent and is approved for use in the treatment of epileptic seizures in adults and children. It is a GABA-receptor agonist and has sodium channel-blocking activity. Studies on the effectiveness of topiramate in treating alcohol dependence demonstrated that oral administration of topiramate led to a decrease in heavy drinking days and alcohol craving, with a concurrent increase in abstinent days and improved liver functions (Johnson et al. *Lancet*, 361:1677-85, 2003). Topiramate has also been shown to be effective in treating binge eating disorder associated with obesity (McElroy et al. *Am. J. Psychiatry* 160:255-261, 2003; McElroy et al. *J. Chn. Psychiatry* 65:1463-9, 2004), and bipolar disorder (Suppes, *J. Chn. Psychopharmacol.* 22:599-609, 2002). More recently, it has been suggested that topiramate may be an effective treatment for obesity.

**Summary of the Invention**

The treatment compositions and methods of the present invention are useful for treating conditions including addictive disorders and neuropsychiatric disorders, such as bipolar disorders, anxiety disorders (including panic disorder, social anxiety disorder, obsessive compulsive disorder, posttraumatic stress disorder, generalized anxiety disorder and specific phobia (American Psychiatric Association, Diagnostic and Statistical Manual of Mental Disorders, 4th edition - Text Revision, 2000)), depression and schizophrenia, that are characterized by neuronal hypersynchrony. The inventive compositions and methods may be employed to reduce neuronal hypersynchrony associated with addictive and/or neuropsychiatric disorders without suppressing neuronal excitability, thereby avoiding the unwanted side effects often associated with agents currently employed for the treatment of addictive disorders and neuropsychiatric disorders.

Addictive disorders include eating disorders (including obesity and binge eating), alcoholism, sexual addiction, addiction to narcotics and smoking, addiction to exercise and gambling. As used herein, the term "addictive disorder" is defined as a disorder characterized by an uncontrollable compulsion to repeat a behavior regardless of its consequences. The inventive compositions and methods may be employed to reduce neuronal hypersynchrony associated with such conditions without suppressing neuronal excitability, thereby avoiding unwanted side effects.

The methods and compositions disclosed herein generally involve non-synaptic mechanisms and modulate, generally reduce, the synchronization of neuronal population activity. The synchronization of neuronal population activity is modulated by
manipulating anionic concentrations and gradients in the central and/or peripheral nervous systems. More specifically, the inventive compositions are capable of reducing the effective amount, inactivating, and/or inhibiting the activity of a Na⁺-K⁺-2Cl⁻ (NKCC) co-transporter. Especially preferred treatment agents of the present invention, exhibit a high degree of NKCC co-transporter antagonist activity in cells of the central and/or peripheral nervous system, e.g., glial cells, Schwann cells and/or neuronal cell populations, and exhibit a lesser degree of activity in renal cell populations. In one embodiment, the inventive compositions are capable of reducing the effective amount, inactivating, and/or inhibiting the activity of the co-transporter NKCC1. NKCC1 antagonists are especially preferred treatment agents for use in the inventive methods.

NKCC co-transporter antagonists that may be usefully employed in the inventive treatment compositions include, but are not limited to, CNS-targeted NKCC co-transporter antagonists such as furosemide, bumetanide, ethacrynic acid, torsemide, azosemide, muzolimine, piretanide, tripamide and the like, as well as thiazide and thiazide-like diuretics, such as bendroflumethiazide, benztiazide, chlorothiazide, hydrochlorothiazide, hydroflumethiazide, methylclothiazide, polythiazide, trichlorothiazide, chlorothalidone, indapamide, metolazone and quinethazone, together with analogs and functional derivatives of such components.

Analogs of CNS-targeted NKCC co-transporter antagonists such as furosemide, bumetanide, piretanide, azosemide and torsemide that may be usefully employed in the inventive compositions and methods include those provided below as Formulas I-VIII. The inventors believe that such analogs have increased lipophilicity and reduced diuretic effects compared to the CNS-targeted NKCC co-transporter antagonists from which they are derived and thus result in fewer undesirable side effects when employed in the inventive treatment methods.

In certain embodiments, compounds according to formula I, II, III, IV, V and/or VI are provided:

![Chemical Structure Image]
or a pharmaceutically acceptable salt, solvate, tautomer or hydrate thereof,
wherein

R₁ is not present, H, O or S;

R₂ is not present, H or when R₁ is O or S, R₂ is selected from the group consisting of
hydrogen, alkyl, aralkyl, aryl, alkylaminodialkyl, alkylcarbonylaminodialkyl,
alkyloxycarbonylalkyl, alkylcarbonyloxyalkyl, alkylaldehyde, alkylketoalkyl, alkylamide,
alkarylamine, arylamine, an alkylammonium group, alkylcarboxylic acid, alkylheteroaryl,
alcohol, a biocompatible polymer such as alkyl(polyalkyloxy)alkylhydroxyl, a
polyethylene glycol (PEG), a polyethylene glycol ester (PEG ester) and a polyethylene
glycol ether (PEG ether), methoxyalkyl, methoxyalkaryl, methylthioalkyl and
methylthioalkaryl, unsubstituted or substituted, and when R₁ is not present, R₂ is selected
from the group consisting of hydrogen, N,N-dialkylamine, N,N-dialkylamine, N,N-
diarylamine, N-alkyl-N-alkarylamine, N-alkyl-N-arylamino, N-alkaryl-N-arylamino,
unsubstituted or substituted;

R₃ is selected from the group consisting of aryl, halo, hydroxy, alkoxy, and
aryloxy, unsubstituted or substituted; and

R₄ and R₅ are each independently selected from the group consisting of hydrogen,
alkylaminodialkyl, carbonylalkyl, carbonylalkaryl, carbonylaryl, and salts thereof such as
sodium, potassium, calcium, ammonium, trialkylammonium and tetraalkylammonium
salts, with the following provisos in some embodiments: R₃ of formula I is not phenyloxy
when R₁ is O and R₂, R₄ and R₅ are H, more specifically, in some embodiments, the
compound of formula I is not bumetanide; R₃ of formula III is not Cl, when R₁ is O and
R₂, R₄ and R₅ are H, more specifically, in some embodiments, the compound of formula
III is not furosemide; R₂ of formula III is not methyl when R₁ is O, R₃ is Cl, and R₄ and
R₅ are H, more specifically, in some embodiments, the compound of formula III is not
furosemide methyl ester; R₃ of formula V is not phenyloxy when R₁ is O and R₂, R₄ and
R₅ are H, more specifically, in some embodiments, the compound of formula V is not
piretanide.

Further embodiments of the present invention provide compounds according to
formula VII:
or a pharmaceutically acceptable salt, solvate, tautomer or hydrate thereof, wherein

R₃, R₄ and R₅ are defined above; and

R₆ is selected from the group consisting of alkylxocarbonylalkyl, alkylaminocarbonylalkyl, alkylaminodialkyl, alkylhydroxy, a biocompatible polymer such as alkylxoy(polyalkylox)alkylhydroxyl, a polyethylene glycol (PEG), a polyethylene glycol ester (PEG ester) and a polyethylene glycol ether (PEG ether), methylxalkyl, methoxyalkaryl, methylthioalkyl and methylthioalkaryl, unsubstituted or substituted, with the proviso that, in some embodiments, R₃ of formula VII is not Cl, when R₄, R₅ and R₆ are H, more specifically, in some embodiments, the compound of formula VII is not azosemide.

Yet further embodiments of the present invention provide compounds according to formula VIII:

or a pharmaceutically acceptable salt, solvate, tautomer or hydrate thereof, wherein

R₇ is not present or selected from the group consisting of hydrogen, alkylxocarbonylalkyl, alkylaminocarbonylalkyl, alkylaminodialkyl, alkylhydroxy, a biocompatible polymer such as alkylxoy(polyalkylox)alkylhydroxyl, a polyethylene
glycol (PEG), a polyethylene glycol ester (PEG ester) and a polyethylene glycol ether (PEG ether), methylxoyalkyl, methylxoyalkaryl, methylthioalkyl and methylthioalkaryl, unsubstituted or substituted; and

X' is a halide such as bromide, chloride, fluoride, iodide or an anionic moiety such as mesylate or tosylate; alternatively, X' is not present and the compound forms an “inner” or zwitterionic salt (where R₇ is H), with the proviso that, in some embodiments, R₇ is always present and X' is not present. More specifically, in some embodiments, the compound of formula VIII is not torsemide.

Embodiments of the present invention provide prodrugs capable of passage across the blood-brain barrier comprising a compound of formula I, II, III, IV, V, VI, VII and/or VIII, or a pharmaceutically acceptable salt, solvate, tautomer or hydrate thereof. In some embodiments, the compound of the prodrug is provided in an amount effective for regulating a CNS disorder. In particular embodiments, the CNS disorder is epilepsy, anxiety, neuropathic pain, neural function, drug addiction/physical dependence and/or migraines.

Embodiments of the present invention provide a pharmaceutical composition comprising a compound of formula I, II, III, FV, V, VI, VII and/or VIII, or a pharmaceutically acceptable salt, solvate, tautomer, hydrate or combination thereof and a pharmaceutically acceptable carrier, excipient or diluent. In certain embodiments, the compound of the pharmaceutical composition is present in an amount effective for regulating a CNS disorder. In particular embodiments, the CNS disorder is an addictive disorder, a neuropsychiatric disorder or neuropathic pain.

Embodiments of the present invention provide uses of the compounds described herein for the preparation of a medicament for treating and/or preventing a CNS disorder selected from the group consisting of: addictive disorders, neuropsychiatric disorders and neuropathic pain.

In one embodiment, the level of diuresis that occurs following administration of an effective amount of an analog provided below as Formula I-VIII, is less than 99%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20% or 10% of that which occurs following administration of an effective amount of the parent molecule from which the analog is derived. For example, the analog may be less diuretic than the parent molecule when administered at the same mg/kg dose. Alternatively, the analog may be more potent than the parent molecule from which it is derived, so that a smaller dose of the analog is required for effective relief of symptoms, thereby eliciting less of a diuretic effect.
Similarly, the analog may have a longer duration of effect in treating disorders than the parent molecule, so that the analog may be administered less frequently than the parent molecule, thus leading to a lower total diuretic effect within any given period of time.

Other treatment agents that may be usefully employed in the inventive compositions and methods include, but are not limited to: antibodies, or antigen-binding fragments thereof, that specifically bind to NKCC1; soluble NKCC1 ligands; small molecule inhibitors of NKCC1; anti-sense oligonucleotides to NKCC1; NKCC1-specific small interfering RNA molecules (siRNA or RNAi); and engineered soluble NKCC1 molecules. Preferably, such antibodies, or antigen-binding fragments thereof, and small molecule inhibitors of NKCC1 specifically bind to the domains of NKCC1 involved in bumetanide binding, as described, for example, in Haas and Forbush II, *Annu. Rev. Physiol.* 62:515-534, 2000. The polypeptide sequence for human NKCC1 is provided in SEQ ID NO: 1, with the corresponding cDNA sequence being provided in SEQ ID NO: 2.

As the methods and treatment agents of the present invention employ "non-synaptic" mechanisms, little or no suppression of neuronal excitability occurs. More specifically, the inventive treatment agents cause little (less than a 1% change compared to pre-administration levels) or no suppression of action potential generation or excitatory synaptic transmission. In fact, a slight increase in neuronal excitability may occur upon administration of certain of the inventive treatment agents. This is in marked contrast to conventional anti-epileptic drugs currently used in the treatment of neuropathic pain, which do suppress neuronal excitability. The methods and treatment agents of the present invention affect the synchronization, or relative synchrony, of neuronal population activity. Preferred methods and treatment agents modulate the extracellular anionic chloride concentration and/or the gradients in the central or peripheral nervous system to reduce neuronal synchronization, or relative synchrony, without substantially affecting neuronal excitability.

In one aspect, the present invention relates to methods and agents for relieving neuropathic pain, or the abnormal perception of pain, by affecting or modulating spontaneous hypersynchronized bursts of neuronal activity and the propagation of action potentials or conduction of impulses in certain cells and nerve fibers of the peripheral nervous system, for example, primary sensory afferent fibers, pain fibers, dorsal horn neurons, and supraspinal sensory and pain pathways.
In another aspect, the present invention related to methods and agents for treating, ameliorating and/or preventing, neuropsychiatric disorders and addictive disorders. In one aspect, the present invention relates to methods and agents for treating or preventing neuropsychiatric disorders, and addictive and/or compulsive disorders, by affecting or modulating spontaneous hypersynchronized bursts of neuronal activity and the propagation of action potentials or conduction of impulses in certain cells and nerve fibers of the peripheral nervous system, for example, primary sensory afferent fibers, pain fibers, dorsal horn neurons, and supraspinal sensory and pain pathways.

The inventive treatment agents may be employed in combination with other, known, treatment agents, such as those presently used in the treatment of neuropsychiatric disorders. One of skill in the art will appreciate that the combination of a treatment agent of the present invention with another, known, treatment agent may involve both synaptic and non-synaptic mechanisms.

Treatment compositions and methods of the present invention may be used therapeutically and episodically following the onset of symptoms or prophylactically, prior to the onset of specific symptoms.

In certain embodiments, the inventive methods for treatment of addictive disorders involve the administration of a treatment agent comprising a diuretic (for example, a loop diuretic such as furosemide, torasemide or bumetanide, or a thiazide or thiazide-like diuretic) in combination with one or more anti-diuretic components, in order to counteract unwanted diuretic effects of the primary treatment agent. Negative side effects that can be avoided by such methods include loss of body water, and depletion of electrolytes (such as potassium, magnesium, calcium and thiamine) and B vitamins. Anti-diuretic components that may be usefully employed in such methods include, for example, anti-diuretic hormones, such as vasopressin, which increases water reabsorption by the kidneys; and salts and electrolytes, which act to replenish ions lost due to diuresis. In a preferred embodiment, the diuretic treatment agent and the anti-diuretic component are combined together in a composition formulated as a liquid beverage, food or food supplement.

In certain embodiments, the treatment agents employed in the inventive methods are capable of crossing the blood brain barrier, and/or are administered using delivery systems that facilitate delivery of the agents to the central nervous system. For example, various blood brain barrier (BBB) permeability enhancers can be used, if desired, to transiently and reversibly increase the permeability of the blood brain barrier to a
treatment agent. Such BBB permeability enhancers may include leukotrienes, bradykinin agonists, histamine, tight junction disrupters (e.g., zonulin), hyperosmotic solutions (e.g., mannitol), cytoskeletal contracting agents, short chain alkylglycerols (e.g., 1-0-pentylglycerol), and others which are currently known in the art.

The above-mentioned and additional features of the present invention, together with the manner of obtaining them, will be best understood by reference to the following more detailed description. All references disclosed herein are hereby incorporated by reference in their entirety as if each was incorporated individually.

**Brief Description of the Drawings**

Figs. IA, IAI, IB, IBI, 1C, ICl and ID show the effect of furosemide on stimulation evoked after discharge activity in rat hippocampal slices.

Figs. 2A - 2R show furosemide blockade of spontaneous epileptiform burst discharges across a spectrum of *in vitro* models.

Figs. 3A - 3H show furosemide blockade of kainic acid-evoked electrical "status epilepticus" in urethane-anesthetized rats, with EKG recordings shown in the upper traces and cortical EEG recordings shown in the bottom traces.

Figs. 4A and 4B show a schematic diagram of ion co-transport under conditions of reduced chloride concentration.

Fig. 5 shows that significantly less freezing was observed in animals treated with either bumetanide or furosemide than in animals receiving vehicle alone in a test of contextual fear conditioning in rats.

Fig. 6 shows baseline startle amplitudes in a fear potentiated startle test in rats.

Fig. 7 shows the amplitude of response in rats on startle alone trials determined immediately following administration of either DMSO alone, bumetanide or furosemide.

Fig. 8 shows the difference score (startle alone - fear potentiated startle) on the test day in rats treated with either DMSO, bumetanide or furosemide.

Fig. 9 shows the startle alone amplitude in rats one week after administration of either DMSO, bumetanide or furosemide.

Fig. 10 shows the difference score in rats one week after administration of either DMSO, bumetanide or furosemide.

Fig. 11 shows the percent difference score (startle alone - fear potentiated startle) on the test day in rats treated with one of the following bumetanide analogs: bumetanide 3-(dimethylaminoproply)ester; bumetanide benzyltrimethylammonium salt; bumetanide...
dibenzylamide; bumetanide cyanomethyl ester; bumetanide N,N-diethylglycolamide ester; bumetanide N,N-dimethylglycolamide ester; bumetanide morpholinodethyl ester; bumetanide pivaxetil ester; bumetanide methyl ester; bumetanide diethylamide; and bumetanide bezyl ester. The vehicle was DMSO.

**Detailed Description of the Invention**

As discussed above, preferred treatment agents and methods of the present invention, for use in treating neuropathic pain, addictive disorders and/or neuropsychiatric disorders, modulate or disrupt the synchrony of neuronal population activity in areas of heightened synchronization by reducing the activity of NKCC co-transporters. As described in detail below and illustrated in the examples, movement of ions and modulation of ionic gradients by means of ion-dependent co-transporters, preferably cation-chloride dependent co-transporters, is critical to regulation of neuronal synchronization. Chloride co-transport function has long been thought to be directed primarily to movement of chloride out of cells. The sodium independent transporter, which has been shown to be neuronally localized, moves chloride ions out of neurons. Blockade of this transporter, such as by administration of the loop diuretic furosemide, leads to hyperexcitability, which is the short-term response to cation-chloride co-transporters such as furosemide. However, the long-term response to furosemide demonstrates that the inward, sodium-dependent movement of chloride ions, mediated by the glial associated Na⁺-K⁺-2Cl⁻ co-transporter NKCC1, plays an active role in blocking neuronal synchronization, without affecting excitability and stimulus-evoked cellular activity. Haglund and Hochman have demonstrated that furosemide is able to block epileptic activity in humans while not affecting normal brain activity (*J. Neurophysiol.* (Feb. 23, 2005) doi:10.1152/ jn.00944.2004). These results provide support for the belief that the inventive methods and compositions may be effectively employed in the treatment of neuropathic pain, neuropsychiatric disorders, and addictive and/or compulsive disorders without giving rise to undesirable side effects often seen with conventional treatments.

As discussed above, the NKCC1 splice variant referred to as NKCCIb is more active than the NKCCIa variant. A central or peripheral nervous system which expresses a few more percentage NKCCIb may thus be more prone to disorders such as neuropathic pain and epilepsy. Similarly, a treatment agent that is more specific for NKCCIb compared to NKCCIa may be more effective in the treatment of such disorders.
The inventive methods may be used for the treatment and/or prophylaxis of neuropathic pain having, for example, the following etiologies: alcohol abuse; diabetes; eosinophilia–myalgia syndrome; Guillain-Barre syndrome; exposure to heavy metals such as arsenic, lead, mercury, and thallium; HIV/AIDS; exposure to anti-HIV/AIDS drugs; malignant tumors; medications including amiodarone, aurothioglucose, cisplatinum, dapsone, stavudine, zalcitabine, didanosine, disulfiram, FK506, hydralazine, isoniazid, metronidazole, nitrofurantoin, paclitaxel, phenytoin and vincristine; monoclonal gammopathies; multiple sclerosis; post-stroke central pain, postherpetic neuralgia; trauma including carpal tunnel syndrome, cervical or lumbar radiculopathy, complex regional pain syndrome, spinal cord injury and stump pain; trigeminal neuralgia; vasculitis; vitamin B6 megadosing; and certain vitamin deficiencies (B12, B1, B6, E).

Neuropsychiatry disorders that may be effectively treated using the inventive methods include, but are not limited to, bipolar disorders, anxiety disorders, panic disorders, depression, schizophrenia, obsessive-compulsive disorders and post-traumatic stress syndrome. Anxiety disorders are classified into several subtypes: Panic Disorder, Social Anxiety Disorder, Obsessive Compulsive Disorder, Posttraumatic Stress Disorder, Generalized Anxiety Disorder and Specific Phobia (American Psychiatric Association, Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, 2000).

The inventive methods may be used for the treatment and/or prophylaxis of addictive and/or compulsive disorders such as: eating disorders, including obesity and binge eating; alcoholism; addiction to narcotics; and smoking.

Compositions that may be effectively employed in the inventive methods are capable of reducing the effective amount, inactivating, and/or inhibiting the activity of a Na⁺-K⁺-2Cl⁻ (NKCC) co-transporter. Preferably such compositions are capable of reducing the effective amount, inactivating, and/or inhibiting the activity of the co-transporter NKCCI. In certain embodiments, the inventive compositions comprise at least one treatment agent selected from the group consisting of: antagonists of NKCCI (including but not limited to, small molecule inhibitors of NKCCI, antibodies, or antigen-binding fragments thereof, that specifically bind to NKCCI and soluble NKCCI ligands); anti-sense oligonucleotides to NKCCI; NKCCI-specific small interfering RNA molecules (siRNA or RNAi); and engineered soluble NKCCI molecules. In preferred embodiments, the treatment agent is selected from the group consisting of: CNS-targeted NKCCI co-transporter antagonists such as furosemide, bumetanide, ethacrynic acid, torsemide, azosemide, muzolimine, piretanide, triamterene and the like; thiazide and
thiazide-like diuretics, such as bendroflumethiazide, benzthiazide, chlorothiazide, hydrochlorothiazide, hydro-fluniethiazide, methylclothiazide, polythiazide, trichlormethiazide, chlorthalidone, indapamide, metolazone and quinethazone; and analogs and functional derivatives of such components.

According to some embodiments, the methods of the present invention employ novel compounds. Thus, any of the R groups as defined herein can be excluded or modified in order to exclude a known compound and/or provide a novel compound. In certain embodiments, the methods disclosed herein employ compounds having the following structures:
or a pharmaceutically acceptable salt, solvate, tautomer or hydrate thereof,
wherein
R₁ is not present, H, O or S;

R₂ is not present, H or when R₁ is O or S, R₂ is selected from the group consisting of hydrogen, alkyl, aralkyl, aryl, alkylaminodialkyl, alkylcarbonylaminodialkyl, alkyloxy carbonylalkyl, alkylcarbonyloxyalkyl, alkylaldehyde, alkylketoalkyl, alkylamide, alkarylamine, arylamide, an alkylammonium group, alkylcarboxylic acid, alkylheteroaryl, alkylhydroxy, a biocompatible polymer such as alkyloxy(polyalkoxy)alkylhydroxyl, a polyethylene glycol (PEG), a polyethylene glycol ester (PEG ester) and a polyethylene glycol ether (PEG ether), methyloxyalkyl, methyloxyaryl, methylthioalkyl and methylthioalkaryl, unsubstituted or substituted, and when R₁ is not present, R₂ is selected from the group consisting of hydrogen, N,N-dialkylamino, N,N-dialkarylamino, N₂N-diarylamino, N-alkyl-N-alkarylarnino, N-alkyl-N-arylamino, N-alkaryl-N-arylamino, unsubstituted or substituted;
R₃ is selected from the group consisting of aryl, halo, hydroxy, alkoxy, and arlyloxy, unsubstituted or substituted; and

R₄ and R₅ are each independently selected from the group consisting of hydrogen, alkylaminodialkyl, carbonylalkyl, carbonylalkaryl, carbonylaryl, and salts thereof such as sodium, potassium, calcium, ammonium, trialkylarylammonium and tetraalkylammonium salts, with the following provisos in some embodiments: R₃ of formula I is not phenyloxy when R₁ is O and R₂, R₄ and R₅ are H, more specifically, in some embodiments, the compound of formula I is not bumetanide; R₃ of formula III is not Cl, when R₁ is O and R₂, R₄ and R₅ are H, more specifically, in some embodiments, the compound of formula III is not furosemide; R₂ of formula III is not methyl when R₁ is O, R₃ is Cl, and R₄ and R₅ are H, more specifically, in some embodiments, the compound of formula III is not furosemide methyl ester; R₃ of formula V is not phenyloxy when R₁ is O and R₂, R₄ and R₅ are H, more specifically, in some embodiments, the compound of formula V is not piretanide.

In some embodiments of the present invention, the compound of formula I can be bumetanide, bumetanide aldehyde, bumetanide methyl ester, bumetanide cyanomethyl ester, bumetanide ethyl ester, bumetanide isoamyl ester, bumetanide octyl ester, bumetanide benzyl ester, bumetanide dibenzylamide, bumetanide diethylamide, bumetanide morpholinoethyl ester, bumetanide 3-(dimethylaminopropyl) ester, bumetanide N,N-diethylglycolamido ester, bumetanide N,N-dimethylglycolamido ester, bumetanide pivaxetil ester, bumetanide propaxetil ester, bumetanide methoxy(polyethyleneoxy)n-i-ethyl ester, bumetanide benzyltrimethylammonium salt and bumetanide cetyltrimethylammonium salt. In particular embodiments, the compound is not bumetanide.

In other embodiments of the present invention, the compound of formula I can be bumetanide [-((C=O)-SH) thioacid, bumetanide S-methyl thioester, bumetanide S-cyanomethyl thioester, bumetanide S-ethyl thioester, bumetanide S-isoamyl thioester, bumetanide S-octyl thioester, bumetanide S-benzyl thioester, bumetanide S-(morpholinoethyl) thioester, bumetanide S-[3-(dimethylaminopropyl)] thioester, bumetanide S-(N,N-diethylglycolamido) thioester, bumetanide S-(N,N-dimethylglycolamido) thioester, bumetanide S-pivaxetil thioester, bumetanide S-propaxetil thioester, bumetanide S-[methoxy(polyethyleneoxy)n-i-ethyl] thioester, bumetanide [-((C=O)-S⁻) benzyltrimethyl-ammonium thioacid salt and bumetanide [-((C=O)-S⁻)] cetyltrimethylammonium thioacid salt.
In some embodiments of the present invention, the compound of formula II can be metastable bumetanide \[-(C=S)-OH\] thioacid, bumetanide O-methyl thioester, bumetanide O-cyanomethyl thioester, bumetanide O-ethyl thioester, bumetanide O-isopropyl thioester, bumetanide O-octyl thioester, bumetanide O-benzyl thioester, bumetanide O-(morpholinoethyl) thioester, bumetanide O-[3-(dimethylaminopropyl)] thioester, bumetanide O-(N,N-diethylglycolamido) thioester, bumetanide, O-(N,N-dimethylglycolamido) thioester, bumetanide O-pivaxetil thioester, bumetanide O-propaxetil thioester, bumetanide O-[methoxy(polyethyleneoxy)\_n-ethyl] thioester, bumetanide \[-(C=S)-O\^-] benzyltrimethylammonium thioacid salt and bumetanide \[-(C=S)-O\^-] cetyltrimethylammonium thioacid salt.

In some embodiments of the present invention, the compound of formula II can be bumetanide thioaldehyde, bumetanide \[-(C=S)-SH\] dithioacid, bumetanide methyl dithioester, bumetanide cyanomethyl dithioester, bumetanide ethyl dithioester, bumetanide isoamyl dithioester, bumetanide octyl dithioester, bumetanide benzyl dithioester, bumetanide dibenzylthioamide, bumetanide diethylthioamide, bumetanide morpholinoethyl dithioester, bumetanide 3-(dimethylaminopropyl) dithioester, bumetanide N,N-diethylglycolamido dithioester, bumetanide N,N-dimethylglycolamido dithioester, bumetanide pivaxetil dithioester, bumetanide propaxetil dithioester, bumetanide methoxy(polyethyleneoxy)\_n-i-ethyl dithioester, bumetanide benzyltrimethylammonium dithioacid salt and bumetanide cetyltrimethylammonium dithioacid salt.

In other embodiments of the present invention, the compound of formula III can be furosemide, furosemide aldehyde, furosemide methyl ester, furosemide cyanomethyl ester, furosemide ethyl ester, furosemide isoamyl ester, furosemide octyl ester, furosemide benzyl ester, furosemide morpholinoethyl ester, furosemide 3-(dimethylaminopropyl) ester, furosemide N,N-diethylglycolamido ester, furosemide N,N-dimethylglycolamido ester, furosemide pivaxetil ester, furosemide propaxetil ester, furosemide methoxy(polyethyleneoxy)\_n-i-ethyl ester, furosemide benzyltrimethylammonium acid salt and furosemide cetyltrimethylammonium acid salt. In particular embodiments, the compound is not furosemide.

In further embodiments of the present invention, the compound of formula III can be furosemide \[-(C=O)-SH\] thioacid, furosemide S-methyl thioester, furosemide S-cyanomethyl thioester, furosemide S-ethyl thioester, furosemide S-isoamyl thioester, furosemide S-octyl thioester, furosemide S-benzyl thioester, furosemide S-
(morpholinoethyl) thioester, furosemide S-[3-(dimethylaminopropyl)] thioester, furosemide S-(N,N-diethylglycolamido) thioester, furosemide S-(N,N-dimethylglycolamido) thioester, furosemide S-pivaxetil thioester, furosemide S-propaxetil thioester, furosemide S-[methoxy(polyethyleneoxy),i-ethyl] thioester, furosemide [-\((\text{C}=\text{O})\text{-S}\)] benzyltrimethylammonium thioacid salt and furosemide [-\((\text{C}=\text{O})\text{-S}\)] cetyltrimethylammonium thioacid salt.

In other embodiments of the present invention, the compound of formula IV can be metastable furosemide \[-(\text{C}=\text{S})\text{-OH}\] thioacid, furosemide O-methyl thioester, furosemide O-cyanomethyl thioester, furosemide O-ethyl thioester, furosemide O-isoamyl thioester, furosemide O-octyl thioester, furosemide O-benzyl thioester, furosemide O-(morpholinoethyl) thioester, furosemide O-[3-(dimethylaminopropyl)] thioester, furosemide O-(N,N-diethylglycolamido) thioester, furosemide O-(N,N-dimethylglycolamido) thioester, furosemide O-pivaxetil thioester, furosemide O-propaxetil thioester, furosemide O-[methoxy(polyethyleneoxy),i-ethyl] thioester, furosemide \[-(\text{C}=\text{S})\text{-O}^+\] benzyltrimethyl-ammonium thioacid salt and furosemide \[-(\text{C}=\text{S})\text{-O}^-\] cetyltrimethylammonium thioacid salt.

In further embodiments of the present invention, the compound of formula IV can be furosemide thioaldehyde, furosemide \[-(\text{C}=\text{S})\text{-SH}\] dithioacid, furosemide methyl dithioester, furosemide cyanomethyl dithioester, furosemide ethyl dithioester, furosemide isoamyl dithioester, furosemide octyl dithioester, furosemide benzyl dithioester, furosemide dibenzylthioamide, furosemide diethylthioamide, furosemide morpholinoethyl dithioester, furosemide 3-(dimethylaminopropyl) dithioester, furosemide N,N-diethylglycolamido dithioester, furosemide N,N-dimethylglycolamido dithioester, furosemide pivaxetil dithioester, furosemide propaxetil dithioester, furosemide methoxy(polyethyleneoxy),i-ethyl dithioester, furosemide benzyltrimethylammonium dithioacid salt and furosemide cetyltrimethylammonium dithioacid salt.

In still further embodiments of the present invention, the compound of formula V can be piretanide, piretanide aldehyde piretanide methyl ester, piretanide cyanomethyl ester, piretanide ethyl ester, piretanide isoamyl ester, piretanide octyl ester, piretanide benzyl ester, piretanide dibenzylamide, piretanide diethylamide, piretanide morpholinoethyl ester, piretanide 3-(dimethylaminopropyl) ester, piretanide N,N-diethylglycolamide ester, piretanide dimethylglycolamide ester, piretanide pivaxetil ester, piretanide propaxetil ester, piretanide methoxy(polyethyleneoxy),i-ethyl ester, piretanide...
benzyltrimethylammonium salt and piretanide cetyltrimethylammonium salt. In particular embodiments, the compound is not piretinide.

In some embodiments of the present invention, the compound of formula V can be piretanide [-\((C=O)\)-SH] thioacid, piretanide S-methyl thioester, piretanide S-cyanomethyl thioester, piretanide S-ethyl thioester, piretanide S-isoamyl thioester, piretanide S-octyl thioester, piretanide S-benzyl thioester, piretanide S-(morpholinoethyl) thioester, piretanide S-[3-(dimethylaminopropyl)] thioester, piretanide S-(N,N-diethylglycolamido) thioester, piretanide S-(N,N-dimethylglycolamido) thioester, piretanide S-pivaxetil thioester, piretanide S-propaxetil thioester, piretanide S-[methoxy(polyethyleneoxy)\_n\_i-ethyl] thioester, piretanide [-\((C=O)\)-S'] benzyltrimethylammonium thioacid salt and piretanide [-\((C=O)\)-S'] cetyltrimethylammonium thioacid salt.

In further embodiments of the present invention, the compound of formula VI can be metastable piretanide [-\((C=S)\)-OH] thioacid, piretanide O-methyl thioester, piretanide O-cyanomethyl thioester, piretanide O-ethyl thioester, piretanide O-isoamyl thioester, piretanide O-octyl thioester, piretanide O-benzyl thioester, piretanide O-(morpholinoethyl) thioester, piretanide O-[3-(dimethylaminopropyl)] thioester, piretanide O-(N,N-diethylglycolamido) thioester, piretanide O-(N,N-dimethylglycolamido) thioester, piretanide O-pivaxetil thioester, piretanide O-propaxetil thioester, piretanide O-[methoxy(polyethyleneoxy)\_n\_i-ethyl] thioester, piretanide [-\((C=S)\)-O'] benzyltrimethylammonium thioacid salt and piretanide [-\((C=S)\)-O'] cetyltrimethylammonium thioacid salt.

In some embodiments, the compound of formula VI can be piretanide thioaldehyde, piretanide [-\((C=S)\)-SH] dithioacid, piretanide methyl dithioester, piretanide cyanomethyl dithioester, piretanide ethyl dithioester, piretanide isoamyl dithioester, piretanide octyl dithioester, piretanide benzyl dithioester, piretanide dibenzylthioamide, piretanide diethylthioamide, piretanide morpholinoethyl dithioester, piretanide 3-(dimethylaminopropyl) dithioester, piretanide N,N-diethylglycolamido dithioester, piretanide N,N-dimethylglycolamido dithioester, piretanide pivaxetil dithioester, piretanide propaxetil dithioester, piretanide methoxy(polyethyleneoxy)\_n\_i-ethyl dithioester, piretanide benzyltrimethylammonium dithioacid salt and piretanide cetyltrimethylammonium dithioacid salt.

In certain embodiments, the methods disclosed herein employ compounds having the following structure:
or a pharmaceutically acceptable salt, solvate, tautomer or hydrate thereof, wherein

\( R_3, R_4 \) and \( R_5 \) are defined above; and

\( R_6 \) is selected from the group consisting of alkyloxycarbonylalkyl, alkylaminocarbonylalkyl, alkylaminodialkyl, alkylhydroxy, a biocompatible polymer such as alkylxy(polyalkyloxy)alkylhydroxy, a polyethylene glycol (PEG), a polyethylene glycol ester (PEG ester) and a polyethylene glycol ether (PEG ether), methylxyalkyl, methylxyalkaryl, methylthioalkyl and methylthioalkaryl, unsubstituted or substituted, with the proviso that, in some embodiments, \( R_3 \) of formula VII is not Cl, when \( R_4, R_5 \) and \( R_6 \) are H, more specifically, in some embodiments, the compound of formula VII is not azosemide.

In certain embodiments, the compounds of formula VII can be tetrazolyl-substituted azosemides (such as methoxymethyl tetrazolyl-substituted azosemides, methylthiomethyl tetrazolyl-substituted azosemides and N-mPEG350-tetrazolyl-substituted azosemides), azosemide benzyltrimethylammonium salt and/or azosemide cetyltrimethylammonium salt.

In yet further embodiments, the methods disclosed herein employ compounds according to formula VIII:
or a pharmaceutically acceptable salt, solvate, tautomer or hydrate thereof,

wherein

R₇ is not present or selected from the group consisting of hydrogen, alkloxy carbonylalkyl, alkylaminocarbonylalkyl, alkylaminodialkyl, alkylhydroxy, a biocompatible polymer such as alkloxy(polyalkyloxy)alkylhydroxy, a polyethylene glycol (PEG), a polyethylene glycol ester (PEG ester) and a polyethylene glycol ether (PEG ether), methoxyalkyl, methoxyalkaryl, methylthioalkyl and methylthioalkaryl, unsubstituted or substituted; and

X⁻ is a halide such as bromide, chloride, fluoride, iodide or an anionic moiety such as mesylate or tosylate; alternatively, X⁻ is not present and the compound forms an "inner" or zwitterionic salt (where R₇ is H), with the proviso that, in some embodiments, R₇ is always present and X⁻ is not present. More specifically, in some embodiments, the compound of formula VIII is not torsemide.

In some embodiments, the compounds of formula VIII can be pyridine-substituted torsemide quaternary ammonium salts or the corresponding inner salts (zwitterions). Examples include, but are not limited to, methoxymethyl pyridinium torsemide salts, methylthiomethyl pyridinium torsemide salts and N-mPEG350-pyridinium torsemide salts.

Embodiments of the present invention further provide intermediate compounds formed through the synthetic methods described herein to provide the compounds of formula I, II, III, IV, V, VI, VII and/or VIII. The intermediate compounds may possess utility as therapeutic agents for the range of indications described herein and/or reagents for further synthesis methods and reactions.

As noted previously, any of the R groups as defined herein can be excluded from the compounds of the present invention, particularly with reference to denoting novel compounds of the present invention.

The term "aryl" or Ar as used herein refers to an aromatic group, a heteroaryl group or to an optionally substituted aromatic group or heteroaryl group fused to one or more optionally substituted aromatic groups or heteroaryl groups, optionally substituted with suitable substituents including, but not limited to, lower alkyl, lower alkoxy, lower alkylsulfanyl, lower alkylsulfenyl, lower alkylsulfonyl, oxo, hydroxy, mercapto, amino optionally substituted by alkyl, carboxy, tetrazolyl, carbamoyl optionally substituted by alkyl, aminosulfonyl optionally substituted by alkyl, acyl, aroyl, heteroaryl, acyloxy, aroyloxy, heteroaryloxy, alkoxycarbonyl, nitro, cyano, halogen, or lower perfluoroalkyl,
multiple degrees of substitution being allowed. Examples of aryl include, but are not limited to, phenyl, 2-naphthyl, 1-naphthyl, 4-pyridyl and the like.

The term "halo" as used herein refers to bromo, chloro, fluoro or iodo. Alternatively, the term "halide" as used herein refers to bromide, chloride, fluoride or iodide.

The term "hydroxy" as used herein refers to the group -OH.

The term "alkoxy" as used herein alone or as part of another group, refers to an alkyl group, as defined herein, appended to the parent molecular moiety through an oxy group. Representative examples of alkoxy include, but are not limited to, methoxy, ethoxy, propoxy, 2-propoxy, butoxy, tert-butoxy, pentyloxy, hexyloxy and the like.

The terms "alkaryloxy" or "oxyalkaryl" as used herein refers to the group -O-alkyl-aryl wherein Ar is aryl. Examples include, but are not limited to, benzyloxy, oxybenzyl, 2-naphthyloxy and oxy-2-naphthyl.

The term "aryloxy" as used herein refers to the group -ArO wherein Ar is aryl or heteroaryl. Examples include, but are not limited to, phenoxy, benzyl and 2-naphthyl.

The term "amino" as used herein refers to -NH₂ in which one or both of the hydrogen atoms may optionally be replaced by alkyl or aryl or one of each, optionally substituted.

The terms "alkylthio" or "thioalkyl" as used herein alone or as part of another group, refers to an alkyl group, as defined herein, appended to the parent molecular moiety through a sulfur moiety. Representative examples of alkylthio include, but are not limited to, methylthio, thiomethyl, ethylthio, thioethyl, n-propylthio, thio-n-propyl, isopropylthio, thio-isopropyl, n-butylthio, thio-n-butyl and the like.

The terms "arylthio" or "thioaryl" as used herein refers to the group -ArS wherein Ar is aryl. Examples include, but are not limited to, phenylthio, thiophenyl, 2-naphthylthio and thio-2-naphthyl.

The terms "alkarylthio" or "thioalkaryl" as used herein refers to the group -S-alkyl-aryl wherein Ar is aryl. Examples include, but are not limited to, benzylthio, thiobenzyl, 2-naphthylthio and thio-2-naphthyl.

The term "carboxy" as used herein refers to the group -CO₂H.

The term "quaternary ammonium" as used herein refers to a chemical structure having four bonds to the nitrogen with a positive charge on the nitrogen in the "onium" state, i.e., "R₄N⁺" or "quaternary nitrogen," wherein R is an organic substituent such as
alkyl or aryl. The term "quaternary ammonium salt" as used herein refers to the association of the quaternary ammonium cation with an anion.

The term "substituted" as used herein refers to replacement of one or more of the hydrogen atoms of the group replaced by substituents known to those skilled in the art and resulting in a stable compound as described below. Examples of suitable replacement groups include, but are not limited to, alkyl, acyl, alkenyl, alkynyl cycloalkyl, aryl, alkaryl, hydroxy, thio, alkoxy, aryloxy, acyl, amino, amido, carboxy, carboxyalkyl, thiocarboxyalkyl, carboxyaryl, thiocarboxyaryl, halo, oxo, mercapto, sulfinyl, sulfonyl, sulfonamido, amidino, carbamoyl, cycloalkyl, heterocycloalkyl, dialkylaminoalkyl, carboxylic acid, carboxamido, haloalkyl, dihaloalkyl, trihaloalkyl, trihaloalkoxy, alkylthio, aralkyl, alkylsulfonyl, arylthio, amino, alkylamino, dialkylamino, guanidino, ureido, nitro and the like. Substitutions are permissible when such combinations result in compounds stable for the intended purpose. For example, substitutions are permissible when the resultant compound is sufficiently robust to survive isolation to a useful degree of purity from a reaction mixture, and formulation into a therapeutic or diagnostic agent or reagent.

The term "effective amount" or "effective" is intended to designate a dose that causes a relief of symptoms of a disease or disorder as noted through clinical testing and evaluation, patient observation, and/or the like. "Effective amount" or "effective" further can further designate a dose that causes a detectable change in biological or chemical activity. The detectable changes may be detected and/or further quantified by one skilled in the art for the relevant mechanism or process. Moreover, "effective amount" or "effective" can designate an amount that maintains a desired physiological state, i.e., reduces or prevents significant decline and/or promotes improvement in the condition of interest. As is generally understood in the art, the dosage will vary depending on the administration routes, symptoms and body weight of the patient but also depending upon the compound being administered.

The term "solvate" as used herein is intended to refer to a pharmaceutically acceptable solvate form of a specified compound that retains the biological effectiveness of such compound, for example, resulting from a physical association of the compound with one or more solvent molecules. Examples of solvates, without limitation, include compounds of the invention in combination with water, 1-propanol, 2-propanol, ethanol, methanol, DMSO, ethyl acetate, acetic acid, or ethanolamine.
The term "hydrate" as used herein refers to the compound when the solvent is water.

The term "biocompatible polymer" as used herein refers to a polymer moiety that is substantially non-toxic and does not tend to produce substantial immune responses, clotting or other undesirable effects. Accordingly to some embodiments of the present invention, polyalkylene glycol is a biocompatible polymer where, as used herein, polyalkylene glycol refers to straight or branched polyalkylene glycol polymers such as polyethylene glycol, polypropylene glycol, and polybutylene glycol, and further includes the monoalkylether of the polyalkylene glycol. In some embodiments of the present invention, the polyalkylene glycol polymer is a lower alkyl polyalkylene glycol moiety such as a polyethylene glycol moiety (PEG), a polypropylene glycol moiety, or a polybutylene glycol moiety. PEG has the formula \(-\text{HO(CH2CH}_2\theta\text{)}_n\text{H}\), where \(n\) can range from about 1 to about 4000 or more. In some embodiments, \(n\) is 1 to 100, and in other embodiments, \(n\) is 5 to 30. The PEG moiety can be linear or branched. In further embodiments, PEG can be attached to groups such as hydroxyl, alkyl, aryl, acyl or ester. In some embodiments, PEG can be an alkoxy PEG, such as methoxy-PEG (or mPEG), where one terminus is a relatively inert alkoxy group, while the other terminus is a hydroxyl group.

**Synthetic Methods**

The compounds of formulas I-VIII can be synthesized using traditional synthesis techniques well known to those skilled in the art. More specific synthesis routes are described below.

The bumetanide analogs are synthesized by reacting the carboxylic acid moiety of bumetanide with various reagents. For example, bumetanide may undergo esterification via reaction with alcohols, including linear, branched, substituted, or unsubstituted alcohols. Bumetanide may also be alkylated via reaction with suitable substituted and unsubstituted alkyl halides and aryl halides, including chloroacetonitrile, benzyl chloride, \(\text{l-(dimethylamino)propyl chloride, 2-chloro-N,N-diethylacetamide, and the like. PEG-type esters may be formed by alkylation using alkylxy(polyalkyloxy)alkyl halides such as MeO-PEG350-Cl and the like, or alkylxy(polyalkyloxy)alkyl tosylates such as MeO-PEGIOOO-OTs and the like. "Axetil"-type esters may also be formed by alkylation using alkyl halides such as chloromethyl pivalate or chloromethyl propionate. Bumetanide may also undergo amidation by reaction with suitable substituted or unsubstituted alkyl amines.
or aryl amines, either after conversion to the acid chloride or by using an activator, such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). Bumetanide may also be reacted with a quaternary ammonium hydroxide, such as benzyltrimethylammonium hydroxide or cetyltrimethylammonium hydroxide, to form bumetanide quaternary ammonium salts.

As used herein, a cotransporter is electroneutral, moving equal amounts of oppositely charged ionic species from one side of a membrane to another. As used herein, a cation-chloride cotransporter refers to a cotransporter that moves one or several cations with an equal number of chloride ions. Exemplary cation chloride cotransporters include, but are not limited to, the loop diuretic-sensitive Na⁺K⁺2Cl⁻ cotransporter in the brain (NKCC1) and the thiazide-sensitive Na⁺Cl⁻ cotransporter (NCC). Discussions regarding the molecular classification of cation-chloride cotransporters, their physiology, and pharmacology can be found in Mount et al. *J Exp Biol* 201: 2091-2102, 1998 and Russell JM. *Physiol Rev.* 2000 Jan; 80(1):211-76.

The NKCC1 brain-specific cotransporter is an isoform of its kidney analog, NKCC2. Furosemide and bumetanide are classic examples of NKCC antagonists.

The thiazide-sensitive cotransporter is antagonized by thiazide diuretics. Exemplary thiazide diuretics include, but are not limited to, chlorothiazide, hydrochlorothiazide, and benzthiazide.

Modification of the diuretic or diuretic-like compound can include reacting the diuretic or diuretic-like compound with a functional group and/or compound selected from the group consisting of an aluminum hydride, alkyl halide, alcohol, aldehyde, alkaryl halide, mono- and dialkylamine, mono- and dialkylamine, mono- and diarylamine, and quaternary ammonium salt, unsubstituted or substituted, or combinations thereof. Non-limiting examples of compounds that may be used as a starting material are exemplified below.
The compounds of formula I, II, III, IV, V, VI, VII and/or VIII can be synthesized using traditional synthesis techniques well known to those skilled in the art. More specific synthesis routes are described below.
A. Bumetanide Analogs, Thiobumetanide Analogs and Dithiobumetanide Analogs

1. Thiobumetanide and dithiobumetanide

The thiobumetanide analogs are synthesized by reacting the carboxylic acid moiety of bumetanide with various reagents. For example, bumetanide may undergo conversion to the corresponding thioacid by treatment with thionyl chloride to form the corresponding bumetanide acid chloride followed by reaction with sodium hydrogen sulfide to give thiobumetanide [-(C=O)-SH], also known as bumetanide [-(C=O)-SH] thioacid by the methodology of Noble, P. and Tarbell, D. S., Ore. Synth., Coll. Vol. IV, John Wiley & Sons, Inc., New York, 1963, 924-927. See Scheme 1. Thiobumetanide may undergo conversion to the corresponding bumetanide thioacid chloride with thionyl chloride, followed by treatment of the thioacid chloride with sodium hydrogen sulfide to give dithiobumetanide [-(C=S)-SH], also known as bumetanide [-(C=S)-SH] dithioacid by similar methodology. Reaction of bumetanide thioacid chloride with secondary amines will give the corresponding bumetanide thioamides. Bumetanide may also undergo reaction with phosphorous pentasulfide to yield bumetanide dithioacid. For reviews of this body of chemistry, see "Thioacyl Halides", "Thiocarboxylic O-Acid Esters" and "Dithiocarboxylic Acid Esters", all by Glass, R. S. in Science of Synthesis, (Charette, A. B., Ed.), Volume 22, Thieme Chemistry, 2005, Chapters 22.1.2, 22.1.3 and 22.1.4 and references therein. See also "Synthesis of Thioamides and Thiolactams", Schaumann, E., in Comprehensive Organic Synthesis, (Trost, B. M. and Fleming, L., Eds.), Permagon Press, 1991, Volume 6, Chapter 2.4, pp. 450-460 and references therein.
Scheme 1. Synthesis of Thiobumetanide \{Bumetanide \[-(C=O)-SH\] Thioacid\}

2. Bumetanide and S-Thiobumetanide Analogs

The bumetanide analogs are synthesized by reacting the carboxylic acid moiety of bumetanide with various reagents. For example, bumetanide may undergo esterification via reaction with alcohols, including linear, branched, substituted, or unsubstituted alcohols. Bumetanide or thiobumetanide may also be alkylated via reaction with suitable substituted and unsubstituted alkyl halides and alkaryl halides, including chloroacetonitrile, benzyl chloride, 1-(dimethylamino)propyl chloride, 2-chloro-N,N-diethylacetamide, and the like. PEG-type esters may be formed by alkylation using alkyloxy(polyalkyloxy)alkyl halides such as MeO-PEG350-Cl and the like or alkyloxy(polyalkyloxy)alkyl tosylates such as MeO-PEG1000-OTs and the like. "Axetil"-type esters may also be formed by alkylation by using alkyl halides such as chloromethyl pivalate or chloromethyl propionate. Bumetanide may also undergo amidation by reaction with suitable substituted or unsubstituted alkyl amines or aryl amines, either after conversion to the acid chloride or by using an activator, such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). Bumetanide or thiobumetanide
may also be reacted with a quaternary ammonium hydroxide, such as benzyltrimethylammonium hydroxide or cetyltrimethylammonium hydroxide, to form bumetanide or thiobemetanide quaternary ammonium salts. Schemes 2, 3 and 4 present synthesis schemes of some exemplary compounds according to formula I.

Scheme 2. Synthesis of Exemplary Compounds According to Formula I
Scheme 3. Synthesis of Exemplary Compounds According to Formula I

Bumetanide salts, thiobumetanide and S-thiobumetanide esters should readily undergo acid- and base-catalyzed hydrolysis to produce the carboxylic acid containing molecule bumetanide by methods well known in the art (See Yang, W. and Drueckhammer, D. G., J. Amer. Chem. Soc. 2001, 123 (44), 11004-11009 and references therein). (See Scheme 4).
Scheme 4. Hydrolysis of Bumetanide, thiobumetanide and S-thiobumetanide esters

bumetanide salts

\[
\begin{align*}
\text{O-Substituted Thiobumetanide Analogs and Dithiobumetanide Analogs} \\
\text{Bumetanide may undergo conversion to the corresponding thioacid by treatment} \\
\text{with thionyl chloride to form the corresponding acid chloride followed by reaction with} \\
\text{sodium hydroxide or sodium hydrogen sulfide to give metastable O-thiobumetanide and} \\
dithiobumetanide by the methodology of Noble, P. and Tarbell, D. S., \textit{Org. Synth., Coll.} \\
\end{align*}
\]
Scheme 5. Synthesis of Metastable Thiobumetanide \{Bumetanide \[-(\text{C=S})-\text{OH}\] thioacid\}

\[
\begin{align*}
\text{thiobumetanide } Y=\text{O} \quad \text{or} \quad \\
\text{dithiobumetanide } Y=\text{S} \\
\end{align*}
\]

\[
\begin{align*}
\text{thiobumetanide acid chloride} \\
\end{align*}
\]

\[
\begin{align*}
\text{isomeric thiobumetanide alkali metal salt} \\
\end{align*}
\]

\[
\begin{align*}
\text{isomeric thiobumetanide} \quad \text{(bumetanide \[-(\text{C=S})-\text{OH}\] dithioacid)} \quad \text{"metastable"}
\end{align*}
\]

The thiobumetanide analogs are, in turn, synthesized by reacting the thiocarboxylic acid moiety of S-thiobumetanide with various reagents. For example, S-thiobumetanide may undergo esterification via reaction with alcohols and thiols, including linear, branched, substituted, or unsubstituted alcohols and thiols. S-Thiobumetanide may also be alkylated via reaction with suitable substituted and unsubstituted alkyl halides and alkaryl halides, including chloroacetonitrile, benzyl chloride, 1-(dimethylamino)propyl chloride, 2-chloro-N,N-diethylacetamide, and the like. PEG-type esters may be formed by alkylation using alkyl(hydroxy)alkyl halides such as MeO-PEG350-Cl and the like or alkyl(hydroxy)alkyl tosylates such as MeO-PEGlOOO-OTs and the like. "Axetil"-type esters may also be formed by alkylation by using alkyl halides such as chloromethyl pivalate or chloromethyl propionate. S-Thiobumetanide may also be reacted with a quaternary ammonium hydroxide, such as benzyltrimethylammonium hydroxide or cetyltrimethylammonium hydroxide, to form thiobumetanide quaternary ammonium salts. See Schemes 7, 8 and 9, which present some exemplary compounds according to formula II.
Scheme 7. Synthesis of Exemplary Compounds According to Formula II

1a. diithiobumetanide

\[ \text{esterification} \] \[ \text{ROH, H}^+ \]

2a. thiobumetanide alkyl esters

\[ R = \text{methyl, ethyl, propyl, i-propyl, butyl, i-butyl, ...} \]
\[ \text{CH}_2\text{CH}_2\text{(OCH}_2\text{CH}_2)_m\text{Y} \]

3a. bumetanide aryl and heterocyclic thioesters

\[ R = \text{phenyl, benzyl, phenethyl, 2-pyridyl, 3-pyridyl, ...} \]
\[ (m = 0, 1, 2, ...) \]

5a. thiobumetanide quaternary ammonium salts

\[ R = \text{benzyl, cetyl, methyl, ethyl, ...} \]
\[ R' = \text{methyl, ethyl, propyl, ...} \]
\[ R'' = \text{methyl, ethyl, propyl, ...} \]
\[ R''' = \text{methyl, ethyl, propyl, ...} \]
Scheme 8. Synthesis of Exemplary Compounds According to Formula II

Thiobumetanide, thiobumetanide amides, O-thiobumetanide esters and dithiobumetanide esters should readily undergo acid- and base-catalyzed hydrolysis to produce the carboxylic acid containing molecule bumetanide by methods well known in the art (See Yang, W. and Drueckhammer, D. G., J. Amer. Chem. Soc. 2001, 123 (44), 11004-11009 and references therein). For additional reviews of this body of chemistry, see "Thiaoacyl Halides", "Thiocarboxylic O-Acid Esters" and "Dithiocarboxylic Acid Esters", all by Glass, R. S. in Science of Synthesis, (Charette, A. B., Ed.), Volume 22, Thieme Chemistry, 2005, Chapters 22.1.2, 22.1.3 and 22.1.4 and references therein. See

Scheme 9. Hydrolysis of Thiobumetanide, Thiobumetanide amides, O-thiobumetanide Esters and Dithiobumetanide Esters
B. Furosemide Analogs, Thiofurosemide Analogs and Dithiofurosemide Analogs

1. Thiofurosemide and dithiofurosemide

The thiofurosemide analogs are synthesized by reacting the carboxylic acid moiety of furosemide with various reagents. For example, furosemide may undergo conversion to the corresponding thioacid by treatment with thionyl chloride to form the corresponding furosemide acid chloride followed by reaction with sodium hydrogen sulfide to give thiofurosemide [-\((C=O)-SH\)], also known as furosemide [-\((C=O)-SH\)] thioacid by the methodology of Noble, P. and Tarbell, D. S., *Ore. Synth., Coll. Vol. IV*, John Wiley & Sons, Inc., New York, 1963, 924-927. (*See Scheme 10*).

Thiofurosemide may undergo conversion to the corresponding furosemide thioacid chloride with thionyl chloride, followed by treatment of the thioacid chloride with sodium hydrogen sulfide to give dithiofurosemide [-\((C=S)-SH\)], also known as furosemide [-\((C=S)-SH\)] dithioacid by similar methodology. (*See Scheme 10*) Reaction of furosemide thioacid chloride with secondary amines will give the corresponding furosemide thioamides. Furosemide may also undergo reaction with phosphorous pentasulfide to yield furosemide dithioacid. For reviews of this body of chemistry, see "Thioacyl Halides", "Thiocarboxylic O-Acid Esters" and "Dithiocarboxylic Acid Esters", all by Glass, R. S. in Science of Synthesis, (Charette, A. B., Ed.), Volume 22, Thieme Chemistry, 2005, Chapters 22.1.2, 22.1.3 and 22.1.4 and references therein. See also "Synthesis of Thioamides and Thiolactams", Schaumann, E., in Comprehensive Organic Synthesis, (Trost, B. M. and Fleming, L., Eds.), Permagon Press, 1991, Volume 6, Chapter 2.4, pp. 450-460 and references therein.
Scheme 10. Synthesis of Thiofurosemide \{Furosemide [-\(\text{C=O}-\text{SH}\) Thioacid]\}

\[
\begin{align*}
\text{furosemide} & \xrightarrow{\text{SOCl}_2} \text{furosemide acid chloride} \\
\text{thiofurosemide alkali metal salt} & \xrightarrow{\text{HCl}, \text{H}_2\text{O}} \text{thiofurosemide [furosemide [-\(\text{C=O}-\text{SH}\) thioacid]}]
\end{align*}
\]

2. Furosemide and S-Furosemide Analogs

The furosemide analogs are synthesized by methods analogous to those used in the synthesis of the bumetanide analogs. Furosemide may undergo esterification via reaction with alcohols, including linear, branched, substituted, or unsubstituted alcohols. Furosemide or thiofurosemide may also be alkylated via reaction with suitable substituted and unsubstituted alkyl halides and alkaryl halides, including for example, chloroacetonitrile, benzyl chloride, \(l\)-(dimethylamino)propyl chloride, 2-chloro-N,N-diethylacetamide, and the like. PEG-type esters may be formed by alkylation using alkylene(polyalkylene)alkyl halides such as MeO-PEG350-Cl and the like or alkylene(polyalkylene)alkyl tosylates such as MeO-PEG1000-OTs and the like. "Axetil"-type esters may also be formed by alkylation by using alkyl halides such as chloromethyl pivalate or chloromethyl propionate. Furosemide may also undergo amidation by reaction with suitable substituted or unsubstituted alkyl amines or aryl amines, either after conversion to the acid chloride or by using an activator, such as \(1\)-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). Furosemide or thiofurosemide may also be reacted with a quaternary ammonium hydroxide, such as benzyltrimethylammonium hydroxide or cetyltrimethylammonium hydroxide, to form furosemide or thiofurosemide quaternary ammonium salts. Schemes, 11, 12 and 13 present some exemplary compounds according to formula III.
Scheme 1. Synthesis of Exemplary Compounds According to Formula III

1. Furosemide

2. Furosemide alkyl esters
   \( R = \text{methyl, ethyl, propyl, i-propyl, butyl, t-butyl, ...} \)
   \( -\text{CH}_2\text{CH}_2\text{OCOR, } X\text{H}_2\text{N Ar, } \text{or alkylation route} \)

3. Furosemide aryl and heteroaryl esters
   \( R = \text{phenyl, benzyl, phenethyl, 2-pyridyl, 3-pyridyl, ...} \)
   \( (m = 0, 1, 2, ...) \)

4. Furosemide simple amides
   \( R = \text{H, methyl, ethyl, benzyl, ...} \)
   \( R' = \text{H, methyl, ethyl, benzyl, ...} \)

5. Furosemide quaternary ammonium salts
   \( R = \text{benzyl, cetyl, methyl, ethyl, ...} \)
   \( R'' = \text{methyl, ethyl, propyl, ...} \)
   \( R''' = \text{methyl, ethyl, propyl, ...} \)

6. Furosemide "axetil"-type esters
   \( R = \text{H, methyl;} \)
   \( R' = \text{H, methyl, ethyl, t-butyl, ...} \)
Scheme 12. Synthesis of Exemplary Compounds According to Formula III

Thiofurosemide salts and S-thiofurosemide esters should readily undergo acid- and base-catalyzed hydrolysis to produce the carboxylic acid containing molecule furosemide by methods well known in the art (See Yang, W. and Drueckhammer, D. G., J. Amer. Chem. Soc, 2001, 123 (44), 11004-11009 and references therein). (See Scheme 13).
Scheme 13. Hydrolysis of Thiofurosemide salts and S-thiofurosemide Esters

S-thiofurosemide esters (S-furosemide thioesters)

acid- or base-catalyzed hydrolysis

S-thiofurosemide esters

acid- or base-catalyzed hydrolysis

O-substituted Thiofurosemide and Dithiofurosemide Analogs

Furosemide may undergo conversion to the corresponding thioacid by treatment with thionyl chloride to form the corresponding acid chloride followed by reaction with sodium hydroxide or sodium hydrogen sulfide to give O-thiofurosemide and dithiofurosemide by the methodology of Noble, P. and Tarbell, D. S., Org. Synth., Coll. Vol. IV, John Wiley & Sons, Inc., New York, 1963, 924-927. (See Schemes 14 and 15).
Scheme 14. Synthesis of Metastable Thiofurosemide \{Furosemide \[-(C=S)-OH]\] thioacid\}

\[
\begin{align*}
\text{thiofurosemide } Y=O \text{ or } \\
\text{dithiofurosemide } Y=S
\end{align*}
\]

\[
\begin{align*}
\text{thiofurosemide acid chloride}
\end{align*}
\]

\[
\begin{align*}
\text{isomeric thiofurosemide alkali metal salt}
\end{align*}
\]

Scheme 15. Synthesis of Dithiofurosemide \{Furosemide \[-(C=S)-SH]\] Dithioacid\}

\[
\begin{align*}
\text{thiofurosemide}
\end{align*}
\]

\[
\begin{align*}
\text{thiofurosemide acid chloride}
\end{align*}
\]

\[
\begin{align*}
\text{dithiofurosemide alkali metal salt}
\end{align*}
\]

\[
\begin{align*}
\text{dithiofurosemide}
\end{align*}
\]

\[
\begin{align*}
\text{dithiofurosemide (furosemide \[-(C=S)-SH]\] dithioacid)}
\end{align*}
\]
The thiofurosemide analogs are, in turn, synthesized by reacting the thiocarboxylic acid moiety of thiofurosemide with various reagents. For example, thiofurosemide may undergo esterification via reaction with alcohols or thiols, including linear, branched, substituted, or unsubstituted alcohols and thiols. S-Thiofurosemide may also be alkylated via reaction with suitable substituted and unsubstituted alkyl halides and alkaryl halides, including chloroacetonitrile, benzyl chloride, 1-(dimethylamino)propyl chloride, 2-chloro-N,N-diethylacetamide, and the like. PEG-type esters may be formed by alkylation using alkylxy(polyalkyloxy)alkyl halides such as MeO-PEG350-Cl and the like or alkylxy(polyalkyloxy)alkyl tosylates such as MeO-PEG1000-OTs and the like. "Axetil"-type esters may also be formed by alkylation by using alkyl halides such as chloromethyl pivalate or chloromethyl propionate. Thiofurosemide may also be reacted with a quaternary ammonium hydroxide, such as benzyltrimethylammonium hydroxide or cetyltrimethylammonium hydroxide, to form thiofurosemide quaternary ammonium salts. Schemes 14, 15, 16, 17 and 18 present synthesis schemes of some exemplary compounds according to formula IV.
Scheme 16. Synthesis of Exemplary Compounds According to Formula IV

1a thiofurosemide

esterification

ROH, H⁺

estimation

ROH, H⁺

R'-CO₂H + H⁺ + R-CHO

quaternary salt formation

R₂N⁺ OH⁻

2a furosemide alkyl thioesters
R = methyl, ethyl, propyl, i-propyl, butyl, i-butyl, ......,
-CH₂CH₂(OCH₂CH₂)ₙ-Y

3a furosemide aryl and heteroaryl thioesters
R = phenyl, benzyl, phenethyl, 2-pyridyl, 3-pyridyl, ...
(m = 0, 1, 2, ......)

6 furosemide "oxalil"-type thioesters
R = H, methyl;
R’ = R'' = methyl, ethyl, t-butyl,......

5a thiofurosemide quaternary ammonium salts
R = benzyl, cetyl, methyl, ethyl ......
R' = methyl, ethyl, propyl, ......
R'' = methyl, ethyl, propyl, ......
R''' = methyl, ethyl, propyl, ......
Scheme 17. Synthesis of Exemplary Compounds According to Formula IV

Thiofurosemide, thiofurosemide amides and S-thiofurosemide esters should readily undergo acid- and base-catalyzed hydrolysis to produce the carboxylic acid containing molecule furosemide by methods well known in the art (See Yang, W. and Drueckhammer, D. G., J. Amer. Chem. Soc., 2001, 123 (44), 11004-11009 and references therein). For additional reviews of this body of chemistry, see "Thiaoacetyl Halides", "Thiocarboxylic O-Acid Esters" and "Dithiocarboxylic Acid Esters", all by Glass, R. S. in Science of Synthesis, (Charette, A. B., Ed.), Volume 22, Thieme Chemistry, 2005, Chapters 22.1.2, 22.1.3 and 22.1.4 and references therein. See also "Synthesis of Thioamides and Thiolactams", Schaumann, E., in Comprehensive Organic Synthesis,
Scheme 18. Hydrolysis of Thiofurosemide, Thiofurosemide Amides and S-thiofurosemide Esters


\[ \text{Thiofurosemide} \xrightarrow{\text{H}_2\text{O}} \text{Thiofurosemide amides} \]

\[ \text{Thiofurosemide esters (furosemide thioesters)} \xrightarrow{\text{H}_2\text{O}} \text{Furosemide} \]

\[ \text{Thiofurosemide} \xrightarrow{\text{H}_2\text{O}} \text{Thiofurosemide esters (furosemide thioesters)} \xrightarrow{\text{H}_2\text{O}} \text{Furosemide} \]

\[ \text{Thiofurosemide amides (furosemide thioamides)} \xrightarrow{\text{H}_2\text{O}} \text{Furosemide} \]
C. Piretanide Analogs and Thiopiretanide Analogs

1. Thiopiretanide and Dithiopiretanide

The piretanide analogs are synthesized by reacting the carboxylic acid moiety of piretanide with various reagents. For example, piretanide may undergo conversion to the corresponding thioacid by treatment with thionyl chloride to form the corresponding piretanide acid chloride followed by reaction with sodium hydrogen sulfide to give thiopiretanide $[-(\text{C}=\text{O})\text{-SH}]$, also known as piretanide $[-(\text{C}=\text{O})\text{-SH}]$ thioacid by the methodology of Noble, P. and Tarbell, D. S., *Org. Synth.*, Coll. Vol. IV, John Wiley & Sons, Inc., New York, 1963, 924-927. *See Scheme 19.* Thiopiretanide may undergo conversion to the corresponding piretanide thioacid chloride with thionyl chloride, followed by treatment of the thioacid chloride with sodium hydrogen sulfide to give dithiopiretanide $[-(\text{C}=\text{S})\text{-SH}]$, also known as piretanide $[-(\text{C}=\text{S})\text{-SH}]$ dithioacid by similar methodology. Reaction of piretanide thioacid chloride with secondary amines will give the corresponding piretanide thioamides. Piretanide may also undergo reaction with phosphozOus pentasulfide to yield piretanide dithioacid. For reviews of this body of chemistry, *see* "Thioacyl Halides", "Thiocarboxylic O-Acid Esters" and "Dithiocarboxylic Acid Esters", all by Glass, R. S. in *Science of Synthesis*, (Charette, A. B., Ed.), Volume 22, Thieme Chemistry, 2005, Chapters 22.1.2, 22.1.3 and 22.1.4 and references therein. See also "Synthesis of Thioamides and Thiolactams", Schaumann, E., in *Comprehensive Organic Synthesis*, (Trost, B. M. and Fleming, L., Eds.), Permagon Press, 1991, Volume 6, Chapter 2.4, pp. 450-460 and references therein.
Scheme 19. Synthesis of Thiopiretanide \{Piretanide \[-(C=O)-SH\] Thioacid\}

2. Piretanide and S-Thiopiretanide Analogs

Piretanide may undergo esterification via reaction with alcohols, including linear, branched, substituted, or unsubstituted alcohols. Piretanide or thiopiretanide may also be alkylated via reaction with suitable substituted and unsubstituted alkyl halides and alkaryl halides, including chloroacetonitrile, benzyl chloride, 1-(dimethylamino)propyl chloride, 2-chloro-N,N-diethylacetamide, and the like. PEG-type esters may be formed by alkylation using alkyl (polyalkyloxy)alkyl halides such as MeO-PEG350-Cl and the like or alkyl (polyalkyloxy)alkyl tosylates such as MeO-PEG1000-OTs and the like. "Axetir'-type esters may also be formed by alkylation by using alkyl halides such as chloromethyl pivalate or chloromethyl propionate. Piretanide may also undergo amidation by reaction with suitable substituted or unsubstituted alkyl amines or aryl amines, either after conversion to the acid chloride or by using an activator, such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). Piretanide or thiopiretanide may also be reacted with a quaternary ammonium hydroxide, such as benzyltrimethylammonium hydroxide or cetyltrimethylammonium hydroxide, to form
piretanide or thiopiretanide quaternary ammonium salts. Schemes 19, 20, 21 and 22 present synthesis schemes of some exemplary compounds according to formula V.

Scheme 20. Synthesis of Exemplary Compounds According to Formula V
Scheme 21. Synthesis of Exemplary Compounds According to Formula V

Thiopiretanide salts and S-thiopiretanide esters should readily undergo acid- and base-catalyzed hydrolysis to produce the carboxylic acid containing molecule bumetanide by methods well known in the art (See Yang, W., Drueckhammer D. G., J. Amer. Chem. Soc., 2001, 123 (44), 11004-11009 and references therein). (See Scheme 22).
Scheme 22. Hydrolysis of Thiopiretanide salts and S-thiopiretanide Esters

![Chemical structures](image)

3. O-Substituted Thiopiretanide Analogs Dithiopiretanide Analogs

Scheme 23. Synthesis of Metastable Thiopiretanide \{Piretanide \[-(C=S)-OH\] thioacid\}

\[
\begin{align*}
\text{thiopiretanide } Y=O \text{ or } dithiopiretanide \ Y=S \\
\end{align*}
\]

\[
\begin{align*}
\text{thiopiretanide acid chloride} \\
\end{align*}
\]

\[
\begin{align*}
\text{isomeric thiopiretanide alkali metal salt} \\
\end{align*}
\]

\[
\begin{align*}
\text{isomeric thiopiretanide} \\
\text{\{piretanide \[-(C=S)-OH\] dithioacid\}} \\
\text{"metastable"}
\end{align*}
\]
Scheme 24. Synthesis of Dithiopiretanide \{Piretanide \[-(C=S)-SH\] Dithioacid\}

The thiopiretanide analogs are synthesized by methods analogous to those used in the synthesis of the piretanide analogs. Specifically, thiopiretanide may undergo esterification via reaction with thiols, including linear, branched, substituted, or unsubstituted thiols. Thiopiretanide may also be alkylated via reaction with suitable substituted and unsubstituted alkyl halides and alkaryl halides, including chloroacetonitrile, benzyl chloride, 1-(dimethylamino)pro pyl chloride, 2-chloro-N,N-diethylacetamide, and the like. PEG-type esters may be formed by alkylation using alkyl(alkoxy(polyalkoxy))alkyl halides such as MeO-PEG350-Cl and the like or alkyl(alkoxy(polyalkoxy))alkyl tosylates such as MeO-PEG1000-OTs and the like. "Axetil"-type thioesters may also be formed by alkylation by using alkyl halides such as chloromethyl pivalate or chloromethyl propionate. Thiopiretanide may also be reacted with a quaternary ammonium hydroxide, such as benzyltrimethylammonium hydroxide or cetyltrimethylammonium hydroxide, to form thiopiretanide quaternary ammonium salts. Schemes 23, 24, 25, 26 and 27 present some exemplary compounds according to formula VI.
Scheme 25. Synthesis of Exemplary Compounds According to Formula VI

1a thiopiretanide

2a piretanide alkylic thioesters

3a piretanide aryl and heteroaryl thioesters

4a piretanide ammonium salts

5a thiopiretanide quaternary ammonium salts
Scheme 26. Synthesis of Exemplary Compounds According to Formula VI

1a thiopti-panide

2a pirenadine alkyl thioesters
R = methyl, ethyl, propyl, i-propyl, butyl, i-butyl, ..... 
-CH₂CH₂(OCH₂CH₂)n=Y

3a pirenadine aryl and heterocyclic thioesters
R = phenyl, benzyl, phenethyl, 2-pyridyl, 3-pyridinyl, ..... 
(m = 0, 1, 2, .....)

4a pirenadine thioamides
R = benzyl, cetyl, methyl, ethyl ..... 
R' = methyl, ethyl, propyl, ..... 
R'' = methyl, ethyl, propyl, ..... 

5a thiopti-panide quaternary ammonium salts
R = H, methyl, ethyl, benzyl ..... 
R' = H, methyl, ethyl, benzyl, ..... 
R'' = methyl, ethyl, propyl, .....

Scheme 27. Hydrolysis of Thiopiretanide, Thiopiretanide Amides and Thiopiretanide Esters
D. Azosemide Analogs

The azosemide analogs are synthesized by the reaction of various reagents with the tetrazolyl moiety of azosemide. Azosemide may undergo hydroxyalkylation with the addition of an aldehyde, whereby a hydroxylalkyl functionality is formed. An alcohol may optionally be reacted along with the aldehyde to obtain an ether. An alkyl thiol may optionally be added with the aldehyde to form a thioether. Azosemide may also be alkylated by the addition of suitable alkyl halides or alkaryl halides, including alkyl or alkaryl halides comprising an ether or thioether linkage, such as methyl chloromethyl ether and benzyl chloromethyl thioether. PEG-type ethers may be formed by alkylation using alkyl(oxy(polyalkyloxy)alkyl halides such as MeO-PEG350-Cl and the like or alkyl(oxy(polyalkyloxy)alkyl tosylates such as MeO-PEG1000-OTs and the like. "Axetir"-type analogs may also be formed via addition of alkyl or alkaryl halides, such as chloromethyl pivalate or chloromethyl propionate. Azosemide may also be reacted with a quaternary ammonium salt, such as benzyltrimethylammoniumbromide and base such as sodium hydroxide or cetyltrimethylammonium bromide and base such as sodium hydroxide, in order to form an azosemide quaternary ammonium salt. Scheme 28 below presents a synthesis scheme of some exemplary compounds according to formula VII.
Scheme 28. Synthesis of Exemplary Compounds According to Formula VII

E. Torsemide Analogs

The torsemide (also known as torasemide) analogs are synthesized by the reaction of various reagents with the pyridine moiety of torsemide. Torsemide may undergo alkylation by the addition of suitable alkyl or alkaryl halides, including benzyl chloride, to form N-substituted quaternary ammonium salts. Alkyl halides and alkaryl halides comprising an ether linkage, including methyl chloromethyl ether and benzyl chloromethyl ether, may be used to form N-substituted ether quaternary ammonium salts.
Alkyl halides and alkaryl halides comprising a thioether linkage, including methyl chloromethyl thioether and benzyl chloromethyl thioether, may be used to form N-substituted thioether quaternary ammonium salts. PEG-type ether-containing quaternary ammonium salts may be formed by alkylation using alkyloxy(polyalkyloxy)alkyl halides such as MeO-PEG350-Cl and the like or alkyloxy(polyalkyloxy)alkyl tosylates such as MeO-PEG1000-OTs and the like. "AxetiF-type quaternary ammonium salts may also be formed via the addition of alkyl halides such as chloromethyl pivalate or chloromethyl propionate. Scheme 29 below presents a synthesis scheme of some exemplary compounds according to formula VIII.
Scheme 29. Synthesis of Exemplary Compounds According to Formula VIII

The substituted benzoic acids bumetanide, piretanide and furosemide can be selectively reduced to the corresponding bumetanide aldehyde, piretanide aldehyde and furosemide aldehyde using amine-substituted ammonium hydrides such as bis(4-memylpiperazinyl)aluminum hydride by literature methods. See Muraki, M. and Mukiayama, T., Chem. Letters, 1974, 1447; Muraki, M. and Mukiayama, T., Chem. Letters, 1975, 215; and Hubert, T., D., Eyman, D. P. and Wiemer, D. F., J. Org. Chem.,
It is well known that the more lipophilic benzaldehydes readily air-oxidize into the more hydrophilic benzoic acids and that benzaldehydes are also metabolized into the corresponding benzoic acids \textit{in vivo}, via the use of NADPH co-factor and with a number of oxidative P450 enzymes.
Scheme 30. Synthesis of Exemplary Benzaldehyde Analogs of Bumetanide, Piretanide and Furosemide

For reduction procedures used to convert benzoic acids to the corresponding benzaldehydes, see:

The lipophilic thiobenzaldehydes can also be prepared from the corresponding benzaldehydes by treating agents including hydrogen sulfide and diphosphorus...
pentasulfide (See Smith, M. B. and March, J., March's Advanced Organic Chemistry, Fifth Edition, 2001, John Wiley & Sons, Inc., New York, Part 2, Chapter 16, pp. 1185-1186. C. Sulfur Nucleophiles, Section 16-10, The Addition of H₂S and Thiols to Carbonyl Compounds.) (See Scheme 31). In turn these thiobenzaldehydes are readily converted back into the corresponding benzaldehydes under hydrolytic conditions. It is well known that the more lipophilic benzaldehydes readily air-oxidize into the more hydrophilic benzoic acids and that benzaldehydes are also metabolized into the corresponding benzoic acids in vivo, via the use of NADPH co-factor and with a number of oxidative P450 enzymes. A similar mechanism can be applied for the conversions of thiobenzaldehydes to thiobenzoic acids and then benzoic acids.
Scheme 31. Synthesis of Exemplary Thiobenzaldehyde Analogs of Bumetanide, Piretanide and Furosemide

bumetanide aldehyde

bumetanide thioaldehyde

K_1 = \ldots, R_2 = H, R_3 = O-aryl, R_4 = R_5 = H

furosemide aldehyde

furosemide thioaldehyde

R_1 = \ldots, R_2 = H, R_3 = \text{halide}, R_4 = R_5 = H

piretanide aldehyde

piretanide thioaldehyde

R_1 = \ldots, R_2 = H, R_3 = O-aryl, R_4 = R_5 = H
G. PEG-Type Analogs of Bumetanide, Piretanide and Furosemide and Their Thioacid Counterparts Thioburaetanide, Thiopiretanide, Thiofurosemide Dithiobumetanide, Dithiopiretanide and Dithiofurosemide

The PEG-type esters of bumetanide, furosemide and piretanide may be formed by alkylation using alkyloxy(polyalkyloxy)alkyl halides such as MeO-PEG350-Cl and the like or alkyloxy(polyalkyloxy)alkyl tosylates such as MeO-PEG1000-OTs and the like. (See Scheme 32).
Scheme 32. Synthesis of Exemplary Polyethylene Glycol Esters of Bumetanide, Furosemide and Piretanide

PEG-type esters of thiobumetanide, thiofurosemide and thiopiretanide may be formed by alkylation using alkylxy(polyalkylxy)alkyl halides such as MeO-PEG350-Cl and the like or alkylxy(polyalkylxy)alkyl tosylates such as MeO-PEG1000-OTs and the like. (See Scheme 33).
Scheme 33. Synthesis of Exemplary Polyethylene Glycol Thioesters of Thiobumetanide, Thiofurosemide and Thiopiretanide

The PEG-type esters of dithiobumetanide, dithiofurosemide and dithiopiretanide may be formed by alkylation using alkylxy(polyalkyloxy)alkyl halides such as MeO-PEG350-C1 and the like or alkylxy(polyalkyloxy)alkyl tosylates such as MeO-PEG1000-OTs and the like. (See Scheme 34).
Scheme 34. Synthesis of Exemplary Polyethylene Glycol Dithioesters of Dithiobumetanide, Dithiofurosemide and Dithiopiretanide

PEG-X is X-(CH$_2$)$_n$-(OCH$_2$CH$_2$)$_m$-Y, where X is halo or other leaving group (mesylate "OMs", tosylate "OTs") and Y is OH or an alcohol protecting group such as an alkyl group, an aryl group, an acyl group or an ester group, and where m = 1 - 5 and n = 1 - 100.
H. PEG-Type Analogs of Azosemide and Torsemide

The PEG-type ethers of azosemide and torsemide may be formed by alkylation using alkyloxy(polyalkyloxy)alkyl halides such as MeO-PEG350-Cl and the like, or alkyloxy(polyalkyloxy)alkyl tosylates such as MeO-PEG1000-OTs and the like. (See Scheme 35).

Scheme 35. Synthesis of Exemplary Alkyl Polyethylene Glycol Ethers of Azosemide and Torsemide

PEG-X is X-(CH₂)m(OCH₂CH₃)n-Y, where X is halo or other leaving group (mesylate "OMs", tosylate "OTS") and Y is OH or an alcohol protecting group such as an alkyl group, an aryl group, an acyl group or an ester group, and where m = 1 - 5 and n = 1 - 100.

Starting materials for synthesizing compounds of the present invention can further include compounds described in U.S. Patent No. 3,634,583 to Feit; U.S. Patent No. 3,806,534 to Fiet; U.S. Patent No. 3,058,882 to Struem et al; U.S. Patent No. 4,010,273...
Compounds of the present invention can include isomers, tautomers, zwitterions, enantiomers, diastereomers, racemates or stereochemical mixtures thereof. The term "isomers" as used herein refers to compounds having the same number and kind of atoms, and hence the same molecular weight, but differing with respect to the arrangement or configuration of the atoms in space. Additionally, the term "isomers" includes stereoisomers and geometric isomers. The terms "stereoisomer" or "optical isomer" as used herein refer to a stable isomer that has at least one chiral atom or restricted rotation giving rise to perpendicular dissymmetric planes (e.g., certain biphenyls, allenes, and spiro compounds) and can rotate plane-polarized light. Because asymmetric centers and other chemical structure can exist in some of the compounds of the present invention which may give rise to stereoisomerism, the invention contemplates stereoisomers and mixtures thereof. The compounds of the present invention and their salts can include asymmetric carbon atoms and may therefore exist as single stereoisomers, racemates, and as mixtures of enantiomers and diastereomers. Typically, such compounds will be prepared as a racemic mixture. If desired, however, such compounds can be prepared or isolated as pure stereoisomers, i.e., as individual enantiomers or diastereomers, or as stereoisomer-enriched mixtures. Tautomers are readily interconvertible constitutional isomers and there is a change in connectivity of a ligand, as in the keto and enol forms of ethyl acetoacetate (The present invention includes tautomers of any said compounds.) Zwitterions are inner salts or dipolar compounds possessing acidic and basic groups in the same molecule. At neutral pH, the cation and anion of most zwitterions are equally ionized.

The present invention further provides prodrugs comprising the compounds described herein. The term "prodrug" is intended to refer to a compound that is converted under physiological conditions, by solvolysis or metabolically, to a specified compound that is pharmaceutically/pharmacologically active. The prodrug can be a compound of the present invention that has been chemically derivatized such that: (i) it retains some, all or none of the bioactivity of its parent drug compound, and (ii) it is metabolized in a subject to yield the parent drug compound. The prodrug of the present invention may also be a "partial prodrug" in that the compound has been chemically derivatized such that: (i) it retains some, all or none of the bioactivity of its parent drug compound, and (ii) it is metabolized in a subject to yield a biologically active derivative of the compound.
The prodrugs can be formed utilizing a hydrolyzable coupling to the compounds described herein. A further discussion of prodrugs can be found in Ettmayer et al. *J. Med. Chem.* 47(10):2394-2404 (2004).

Prodrugs of the present invention are capable of passage across the blood-brain barrier and may undergo hydrolysis by CNS esterases to provide the active compound. Further, the prodrugs provided herein may also exhibit improved bioavailability, improved aqueous solubility, improved passive intestinal absorption, improved transporter-mediated intestinal absorption, protection against accelerated metabolism, tissue-selective delivery and/or passive enrichment in the target tissue.

Prodrugs of the present invention can include compounds according to formula I, II, III, IV and/or V described herein. Prodrugs of the present invention can further include bumetanide, bumetanide dibenzylamide, bumetanide diethylamide, bumetanide morpholinoethyl ester, bumetanide 3-(dimethylaminopropyl) ester, bumetanide N,N-diethylglycolamide ester, bumetanide dimethylglycolamide ester, bumetanide pivaxetil ester, furosemide, furosemide ethyl ester, furosemide cyanethyl ester, furosemide benzyl ester, furosemide morpholinoethyl ester, furosemide 3-(dimethylaminopropyl) ester, furosemide N,N-diethylglycolamide ester, furosemide dibenzylamide, furosemide benzyltrimethyl-ammonium salt, furosemide cetyltrimethylammonium salt, furosemide N,N-dimethylglycolamide ester, furosemide pivaxetil ester, furosemide propaxetil ester, piretanide, piretanide methyl ester, piretanide cyanomethyl ester, piretanide benzyl ester, piretanide morpholinoethyl ester, piretanide 3-(dimethylaminopropyl) ester, piretanide N,N-diethylglycolamide ester, piretanide diethylamide, piretanide dibenzylamide, piretanide benzyltrimethylammonium salt, piretanide cetyltrimethylammonium salt, piretanide N,N-dimethylglycolamide ester, piretanide pivaxetil ester, piretanide propaxetil ester, tetrazolyl-substituted azosemides, pyridinium-substituted torsemide salts (also termed pyridine-substituted torsemide quaternary ammonium salts), as well as similar derivatives of indacrinone, and ozolinone. See previously presented schemes.

Moreover, as shown in the previously presented schemes, prodrugs can be formed by attachment of biocompatible polymers, such as those previously described including polyethylene glycol (PEG), to compounds of the present invention using linkages degradable under physiological conditions. See also Schacht, E.H. et al. *Poly(ethylene glycol) Chemistry and Biological Applications*, American Chemical Society, San Francisco, CA 297-315 (1997). Attachment of PEG to proteins can be employed to reduce immunogenicity and/or extend the half-life of the compounds provided herein.
Any conventional PEGylation method can be employed, provided that the PEGylated agent retains pharmaceutical activity.

Compositions of the subject invention are suitable for human and veterinary applications and are preferably delivered as pharmaceutical compositions. Pharmaceutical compositions comprise one or more treatment agents, or a pharmaceutically acceptable salt thereof, and a physiologically acceptable carrier. A pharmaceutically acceptable salt, as used herein, refers to a salt form of a compound permitting its use or formulation as a pharmaceutical and which retains the biological effectiveness of the free acid and base of the specified compound and is not biologically or otherwise undesirable. Examples of such salts are described in *Handbook of Pharmaceutical Salts: Properties, Selection, and Use*, Wermuth, CG. and Stahl, P.H. (eds.), Wiley-Verlag Helvetica Acta, Zurich, 2002 [ISBN 3-906390-26-8]. Examples of such salts include alkali metal salts and addition salts of free acids and bases. Examples of pharmaceutically acceptable salts, include, but are not limited to, sulfates, pyrosulfates, bisulfates, sulfites, bisulfites, phosphates, monohydrogen-phosphates, dihydrogen phosphates, metaphosphates, pyrophosphates, chlorides, bromides, iodides, acetates, propionates, decanoates, caprylates, acrylates, formates, isobutyrate, caprates, heptanoates, propiolates, oxalates, malonates, succinates, suberates, sebacates, fumarates, maleates, butyne-1,4-dioates, hexyne-1,6-dioates, benzoates, chlorobenzoates, methylbenzoates, dinitrobenzoates, hydroxybenzoates, methoxybenzoates, phthalates, xylenesulphonates, phenylacetates, phenylpropionate, phenylbutyrate, citrates, lactates, γ-hydroxybutyrates, glycollates, tartrates, methanesulphonates, ethane sulfonates, propanesulphonates, toluenesulphonates, naphthalene-1-sulphonates, naphthalene-2-sulphonates, and mandelates.

Pharmaceutical compositions of the present invention may also contain other compounds, which may be biologically active or inactive. For example, one or more treatment agents of the present invention may be combined with another agent, in a treatment combination, and administered according to a treatment regimen of the present invention. Such combinations may be administered as separate compositions, combined for delivery in a complementary delivery system, or formulated in a combined composition, such as a mixture or a fusion compound. Additionally, the aforementioned treatment combination may include a BBB permeability enhancer and/or a hyperosmotic agent.
The carriers and additives used for such pharmaceutical compositions can take a variety of forms depending on the anticipated mode of administration. Thus, compositions for oral administration may be, for example, solid preparations such as tablets, sugar-coated tablets, hard capsules, soft capsules, granules, powders and the like, with suitable carriers and additives being starches, sugars, binders, diluents, granulating agents, lubricants, disintegrating agents and the like. Because of their ease of use and higher patient compliance, tablets and capsules represent advantageous oral dosage forms for many medical conditions.

Similarly, compositions for liquid preparations include solutions, emulsions, dispersions, suspensions, syrups, elixirs, and the like with suitable carriers and additives being water, alcohols, oils, glycols, preservatives, flavoring agents, coloring agents, suspending agents, and the like. Typical preparations for parenteral administration comprise the active ingredient with a carrier such as sterile water or parenterally acceptable oil including polyethylene glycol, polyvinyl pyrrolidone, lecithin, arachis oil or sesame oil, with other additives for aiding solubility or preservation may also be included. In the case of a solution, it can be lyophilized to a powder and then reconstituted immediately prior to use. For dispersions and suspensions, appropriate carriers and additives include aqueous gums, celluloses, silicates or oils.

The pharmaceutical compositions according to embodiments of the present invention include those suitable for oral, rectal, topical, nasal, inhalation (e.g., via an aerosol) buccal (e.g., sub-lingual), vaginal, topical (i.e., both skin and mucosal surfaces, including airway surfaces), transdermal administration and parenteral (e.g., subcutaneous, intramuscular, intradermal, intraarticular, intrapleural, intraperitoneal, intrathecal, intracerebral, intracranially, intraarterial, or intravenous), although the most suitable route in any given case will depend on the nature and severity of the condition being treated and on the nature of the particular active agent which is being used. Pharmaceutical compositions of the present invention are particularly suitable for oral, sublingual, parenteral, implantation, nasal and inhalational administration.

Compositions for injection will include the active ingredient together with suitable carriers including propylene glycol-alcohol-water, isotonic water, sterile water for injection (USP), emulPhor™-alcohol-water, cremophor-EL™ or other suitable carriers known to those skilled in the art. These carriers may be used alone or in combination with other conventional solubilizing agents such as ethanol, a glycol, or other agents known to those skilled in the art.
Where the compounds of the present invention are to be applied in the form of solutions or injections, the compounds may be used by dissolving or suspending in any conventional diluent. The diluents may include, for example, physiological saline, Ringer's solution, an aqueous glucose solution, an aqueous dextrose solution, an alcohol, a fatty acid ester, glycerol, a glycol, an oil derived from plant or animal sources, a paraffin and the like. These preparations may be prepared according to any conventional method known to those skilled in the art.

Compositions for nasal administration may be formulated as aerosols, drops, powders and gels. Aerosol formulations typically comprise a solution or fine suspension of the active ingredient in a physiologically acceptable aqueous or non-aqueous solvent. Such formulations are typically presented in single or multidose quantities in a sterile form in a sealed container. The sealed container can be a cartridge or refill for use with an atomizing device. Alternatively, the sealed container may be a unitary dispensing device such as a single use nasal inhaler, pump atomizer or an aerosol dispenser fitted with a metering valve set to deliver a therapeutically effective amount, which is intended for disposal once the contents have been completely used. When the dosage form comprises an aerosol dispenser, it will contain a propellant such as a compressed gas, air as an example, or an organic propellant including a fluorochlorohydrocarbon or fluorohydrocarbon.

Compositions suitable for buccal or sublingual administration include tablets, lozenges and pastilles, wherein the active ingredient is formulated with a carrier such as sugar and acacia, tragacanth or gelatin and glycerin.

Compositions for rectal administration include suppositories containing a conventional suppository base such as cocoa butter.

Compositions suitable for transdermal administration include ointments, gels and patches.

Other compositions known to those skilled in the art can also be applied for percutaneous or subcutaneous administration, such as plasters.

Further, in preparing such pharmaceutical compositions comprising the active ingredient or ingredients in admixture with components necessary for the formulation of the compositions, other conventional pharmacologically acceptable additives may be incorporated, for example, excipients, stabilizers, antiseptics, wetting agents, emulsifying agents, lubricants, sweetening agents, coloring agents, flavoring agents, isotonicity agents, buffering agents, antioxidants and the like. As the additives, there may be
mentioned, for example, starch, sucrose, fructose, dextrose, lactose, glucose, mannitol, sorbitol, precipitated calcium carbonate, crystalline cellulose, carboxymethylcellulose, dextrin, gelatin, acacia, EDTA, magnesium stearate, talc, hydroxypropylmethylcellulose, sodium metabisulfite, and the like.

In further embodiments, the present invention provides kits including one or more containers comprising pharmaceutical dosage units comprising an effective amount of one or more compounds of the present invention.

When aqueous suspensions or elixirs are desired for oral administration, the essential active ingredient therein may be combined with various sweetening or flavoring agents, coloring matter or dyes and, if desired, emulsifying or suspending agents, together with diluents such as water, ethanol, propylene glycol, glycerin and combinations thereof.

The compositions described herein may be administered as part of a sustained release formulation. Such formulations may generally be prepared using well-known technology and administered by, for example, oral, rectal or transdermal delivery systems, or by implantation of a formulation or therapeutic device at one or more desired target site(s). Sustained-release formulations may contain a treatment composition comprising an inventive treatment agent alone, or in combination with a second treatment agent, dispersed in a carrier matrix and/or contained within a reservoir surrounded by a rate controlling membrane. Carriers for use within such formulations are biocompatible, and may also be biodegradable. According to one embodiment, the sustained release formulation provides a relatively constant level of active composition release. According to another embodiment, the sustained release formulation is contained in a device that may be actuated by the subject or medical personnel, upon onset of certain symptoms, for example, to deliver predetermined dosages of the treatment composition. The amount of the treatment composition contained within a sustained release formulation depends upon the site of implantation, the rate and expected duration of release, and the nature of the condition to be treated or prevented.

In certain embodiments, compositions of the present invention for treatment of neuropathic pain and neuropsychiatric disorders are administered using a formulation and a route of administration that facilitates delivery of the treatment composition(s) to the central nervous system. Treatment compositions, such as NKCCI antagonists, may be formulated to facilitate crossing of the blood brain barrier as described above, or may be co-administered with an agent that crosses the blood brain barrier. Treatment compositions may be delivered in liposome formulations, for example, that cross the
blood brain barrier, or may be co-administered with other compounds, such as bradykinins, bradykinin analogs or derivatives, or other compounds, such as SERAPORT™, that cross the blood brain barrier. Alternatively, treatment compositions of the present invention may be delivered using a spinal tap that places the treatment composition directly in the circulating cerebrospinal fluid. For some treatment conditions, there may be transient or permanent breakdowns of the blood brain barrier and specialized formulation of the treatment composition to cross the blood brain barrier may not be necessary. We have determined, for example, that a bolus iv injection of 20 mg furosemide reduces or abolishes both spontaneous interictal activity and electrical stimulation-evoked epileptiform activity in human patients who are refractory to antiepileptic drugs (AEDs) (Haglund & Hochman J Neurophysiol. (Feb. 23, 2005) doi:10.1152/jn.00944.2004).

Routes and frequency of administration of the therapeutic compositions disclosed herein, as well as dosages, vary according to the indication, and from individual to individual, and may be readily determined by a physician from information that is generally available, and by monitoring patients and adjusting the dosages and treatment regimen accordingly using standard techniques. In general, appropriate dosages and treatment regimen provide the active composition(s) in an amount sufficient to provide therapeutic and/or prophylactic benefit. Dosages and treatment regimen may be established by monitoring improved clinical outcomes in treated patients as compared to non-treated patients.

The term "effective amount" or "effective" is intended to designate a dose that causes a relief of symptoms of a disease or disorder as noted through clinical testing and evaluation, patient observation, and/or the like. "Effective amount" or "effective" further can further designate a dose that causes a detectable change in biological or chemical activity. The detectable changes may be detected and/or further quantified by one skilled in the art for the relevant mechanism or process. Moreover, "effective amount" or "effective" can designate an amount that maintains a desired physiological state, i.e., reduces or prevents significant decline and/or promotes improvement in the condition of interest. Therapeutically effective dosages and treatment regimen will depend on the condition, the severity of the condition, and the general state of the patient being treated. Since the pharmacokinetics and pharmacodynamics of the treatment compositions of the present invention vary in different patients, a preferred method for determining a therapeutically effective dosage in a patient is to gradually escalate the dosage and
monitor the clinical and laboratory indicia. For combination therapy, the two or more agents are coadministered such that each of the agents is present in a therapeutically effective amount for sufficient time to produce a therapeutic or prophylactic effect. The term "coadministration" is intended to encompass simultaneous or sequential administration of two or more agents in the same formulation or unit dosage form or in separate formulations. Appropriate dosages and treatment regimen for treatment of acute episodic conditions, chronic conditions, or prophylaxis will necessarily vary to accommodate the condition of the patient.

By way of example, for the treatment to neuropathic pain, furosemide may be administered orally to a patient in amounts of 10-40 mg at a frequency of 1-3 times per day, preferably in an amount of 40 mg three times per day. In an alternative example, bumetanide may be administered orally for the treatment of neuropathic pain in amounts of 1-10 mg at a frequency of 1-3 times per day. One of skill in the art will appreciate that smaller doses may be employed, for example, in pediatric applications.

In further embodiments, bumetanide analogs according to the present invention may be administered in amounts of 1.5 to 6 mg daily, for example 1 tablet or capsule three times a day. In some embodiments, furosemide analogs according to the present invention may be administered in amounts of 60 to 240 mg/day, for example 1 tablet or capsule three times a day. In other embodiments, piretanide analogs according to the present invention may be administered in amounts of 10 to 20 mg daily, for example 1 tablet or capsule once a day. In some embodiments, azosemide analogs according to the present invention may be administered in an amount of 60 mg per day. In other embodiments, torsemide analogs according to the present invention may be administered in amounts of 10 to 20 mg daily, for example 1 tablet or capsule once a day. It should be noted that lower doses may be administered, particularly for IV administration.

Methods and systems of the present invention may also be used to evaluate candidate compounds and treatment regimen for the treatment and/or prophylaxis of neuropathic pain and neuropsychiatric disorders. Various techniques for generating candidate compounds potentially having the desired NKCC1 cotransporter antagonist activity may be employed. Candidate compounds may be generated using procedures well known to those skilled in the art of synthetic organic chemistry. Structure-activity relationships and molecular modeling techniques are useful for the purpose of modifying known NKCC1 antagonists, such furosemide, bumetanide, ethacrinic acid and related compounds, to confer the desired activities and specificities. Methods for screening
Candidate compounds for desired activities are described in U.S. Patents 5,902,732, 5,976,825, 6,096,510 and 6,319,682, which are incorporated herein by reference in their entireties.

Candidate compounds may be screened for NKCC1 antagonist activity using screening methods of the present invention with various types of cells in culture such as glial cells, neuronal cells, renal cells, and the like, or in situ in animal models. Screening techniques to identify chloride cotransporter antagonist activity, for example, may involve altering the ionic balance of the extracellular space in the tissue culture sample, or in situ in an animal model, by producing a higher than "normal" anionic chloride concentration. The geometrical and/or optical properties of the cell or tissue sample subject to this altered ionic balance are determined, and candidate agents are administered. Following administration of the candidate agents, the corresponding geometrical and/or optical properties of the cell or tissue sample are monitored to determine whether the ionic imbalance remains, or whether the cells responded by altering the ionic balances in the extracellular and intracellular space. If the ionic imbalance remains, the candidate agent is likely a chloride cotransporter antagonist. By screening using various types of cells or tissues, candidate compounds having a high level of glial cell chloride cotransporter antagonist activity and having a reduced level of neuronal cell and renal cell chloride cotransporter antagonist activity may be identified. Similarly, effects on different types of cells and tissue systems may be assessed.

Additionally, the efficacy of candidate compounds may be assessed by simulating or inducing a condition, such as neuropathic pain, in situ in an animal model, monitoring the geometrical and/or optical properties of the cell or tissue sample during stimulation of the condition, administering the candidate compound, then monitoring the geometrical and/or optical properties of the cell or tissue sample following administration of the candidate compound, and comparing the geometrical and/or optical properties of the cell or tissue sample to determine the effect of the candidate compound. Testing the efficacy of treatment compositions for relief of neuropathic pain can be carried using well known methods and animal models, such as that described in Bennett, Hosp. Pract. (Off Ed). 33:95-98, 1998.

As discussed above, compositions for use in the inventive methods may comprise a treatment agent selected from the group consisting of: antibodies, or antigen-binding fragments thereof, that specifically bind to NKCC1; soluble ligands that bind to NKCC1;
anti-sense oligonucleotides to NKCC1; and small interfering RNA molecules (siRNA or RNAi) that are specific for NKCC1.

Antibodies that specifically bind to NKCC1 are known in the art and include those available from Alpha Diagnostic International, Inc. (San Antonio, TX 78238). An "antigen-binding site," or "antigen-binding fragment" of an antibody refers to the part of the antibody that participates in antigen binding. The antigen binding site is formed by amino acid residues of the N-terminal variable ("V") regions of the heavy ("H") and light ("L") chains. Three highly divergent stretches within the V regions of the heavy and light chains are referred to as "hypervariable regions" which are interposed between more conserved flanking stretches known as "framework regions," or "FRs." Thus the term "FR" refers to amino acid sequences which are naturally found between and adjacent to hypervariable regions in immunoglobulins. In an antibody molecule, the three hypervariable regions of a light chain and the three hypervariable regions of a heavy chain are disposed relative to each other in three dimensional space to form an antigen-binding surface. The antigen-binding surface is complementary to the three-dimensional surface of a bound antigen, and the three hypervariable regions of each of the heavy and light chains are referred to as "complementarity-determining regions," or "CDRs."

A number of molecules are known in the art that comprise antigen-binding sites capable of exhibiting the binding properties of an antibody molecule. For example, the proteolytic enzyme papain preferentially cleaves IgG molecules to yield several fragments, two of which (the "F(ab)" fragments) each comprise a covalent heterodimer that includes an intact antigen-binding site. The enzyme pepsin is able to cleave IgG molecules to provide several fragments, including the "F(ab')₂" fragment, which comprises both antigen-binding sites. An "Fv" fragment can be produced by preferential proteolytic cleavage of an IgM, IgG or IgA immunoglobulin molecule, but are more commonly derived using recombinant techniques known in the art. The Fv fragment includes a non-covalent $V_H$: $V_L$ heterodimer including an antigen-binding site which retains much of the antigen recognition and binding capabilities of the native antibody molecule (Inbar et al. Proc. Natl. Acad. Sci. USA 69:2659-2662, 1972; Hochman et al. Biochem 75:2706-2710, 1976; and Ehrlich et al. Biochem 79:4091-4096, 1980).

Humanized antibodies that specifically bind to NKCC1 may also be employed in the inventive methods. A number of humanized antibody molecules comprising an antigen-binding site derived from a non-human immunoglobulin have been described, including chimeric antibodies having rodent V regions and their associated CDRs fused to

Modulating the activity of NKCCI may alternatively be accomplished by reducing or inhibiting expression of the polypeptide, which can be achieved by interfering with transcription and/or translation of the corresponding polynucleotide. Polypeptide expression may be inhibited, for example, by introducing anti-sense expression vectors, anti-sense oligodeoxyribonucleotides, anti-sense phosphorothioate oligodeoxyribonucleotides, anti-sense oligoribonucleotides or anti-sense phosphorothioate oligoribonucleotides; or by other means well known in the art. All such anti-sense polynucleotides are referred to collectively herein as "anti-sense oligonucleotides".

The anti-sense oligonucleotides for use in the inventive methods are sufficiently complementary to the NKCCI polynucleotide to bind specifically to the polynucleotide. The sequence of an anti-sense oligonucleotide need not be 100% complementary to the of the polynucleotide in order for the anti-sense oligonucleotide to be effective in the inventive methods. Rather an anti-sense oligonucleotide is sufficiently complementary when binding of the anti-sense oligonucleotide to the polynucleotide interferes with the normal function of the polynucleotide to cause a loss of utility, and when non-specific binding of the oligonucleotide to other, non-target sequences is avoided. The design of appropriate anti-sense oligonucleotides is well known in the art. Oligonucleotides that are complementary to the 5’ end of the message, for example the 5’ untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, oligonucleotides complementary to either the 5’- or 3’-non-translated, non-coding, regions of the targeted polynucleotide may also be employed. Cell permeation and activity of anti-sense oligonucleotides can be enhanced by appropriate chemical modifications, such as the use of phenoxazine-substituted C-5
propynyl uracil oligonucleotides (Flanagan et al., Nat. Biotechnol. 17:48-52, 1999) or 2'-\(\text{O-}(2\text{-methoxy})\) ethyl (2'\text{-MOE})-oligonucleotides (Zhang et al, Nat. Biotechnol. 18:862-867, 2000). The use of techniques involving anti-sense oligonucleotides is well known in the art and is described, for example, in Robinson-Benion et al. (Methods in Enzymol. 254:363-375, 1995) and Kawasaki et al. (Artific. Organs 20:836-848, 1996).

Expression of the NKCCI polypeptide may also be specifically suppressed by methods such as RNA interference (RNAi). A review of this technique is found in Science, 288:1370-1372, 2000. Briefly, traditional methods of gene suppression, employing anti-sense RNA or DNA, operate by binding to the reverse sequence of a gene of interest such that binding interferes with subsequent cellular processes and therefore blocks synthesis of the corresponding protein. RNAi also operates on a post-translational level and is sequence specific, but suppresses gene expression far more efficiently. Exemplary methods for controlling or modifying gene expression are provided in WO 99/49029, WO 99/53050 and WO01/75164, the disclosures of which are hereby incorporated by reference. In these methods, post-transcriptional gene silencing is brought about by a sequence-specific RNA degradation process which results in the rapid degradation of transcripts of sequence-related genes. Studies have shown that double-stranded RNA may act as a mediator of sequence-specific gene silencing (see, for example, Montgomery and Fire, Trends in Genetics, 14:255-258, 1998). Gene constructs that produce transcripts with self-complementary regions are particularly efficient at gene silencing.

It has been demonstrated that one or more ribonucleases specifically bind to and cleave double-stranded RNA into short fragments. The ribonuclease(s) remains associated with these fragments, which in turn specifically bind to complementary mRNA, i.e. specifically bind to the transcribed mRNA strand for the gene of interest. The mRNA for the gene is also degraded by the ribonuclease(s) into short fragments, thereby obviating translation and expression of the gene. Additionally, an RNA-polymerase may act to facilitate the synthesis of numerous copies of the short fragments, which exponentially increases the efficiency of the system. A unique feature of RNAi is that silencing is not limited to the cells where it is initiated. The gene-silencing effects may be disseminated to other parts of an organism.

The NKCCI polynucleotide may thus be employed to generate gene silencing constructs and/or gene-specific self-complementary, double-stranded RNA sequences that can be employed in the inventive methods using delivery methods known in the art. A
gene construct may be employed to express the self-complementary RNA sequences. Alternatively, cells may be contacted with gene-specific double-stranded RNA molecules, such that the RNA molecules are internalized into the cell cytoplasm to exert a gene silencing effect. The double-stranded RNA must have sufficient homology to the NKCCI gene to mediate RNAi without affecting expression of non-target genes. The double-stranded DNA is at least 20 nucleotides in length, and is preferably 21-23 nucleotides in length. Preferably, the double-stranded RNA corresponds specifically to a polynucleotide of the present invention. The use of small interfering RNA (siRNA) molecules of 21-23 nucleotides in length to suppress gene expression in mammalian cells is described in WO 01/75164. Tools for designing optimal inhibitory siRNAs include that available from DNAengine Inc. (Seattle, WA).

One RNAi technique employs genetic constructs within which sense and anti-sense sequences are placed in regions flanking an intron sequence in proper splicing orientation with donor and acceptor splicing sites. Alternatively, spacer sequences of various lengths may be employed to separate self-complementary regions of sequence in the construct. During processing of the gene construct transcript, intron sequences are spliced-out, allowing sense and anti-sense sequences, as well as splice junction sequences, to bind forming double-stranded RNA. Select ribonucleases then bind to and cleave the double-stranded RNA, thereby initiating the cascade of events leading to degradation of specific mRNA gene sequences, and silencing specific genes.

For in vivo uses, a genetic construct, anti-sense oligonucleotide or RNA molecule may be administered by various art-recognized procedures (see, e.g., Rolland, CHt. Rev. Therap. Drug Carrier Systems /5:143-198, 1998, and cited references). Both viral and non-viral delivery methods have been used for gene therapy. Useful viral vectors include, for example, adenovirus, adeno-associated virus (AAV), retrovirus, vaccinia vims and avian poxvirus. Improvements have been made in the efficiency of targeting genes to tumor cells with adenoviral vectors, for example, by coupling adenovirus to DNA-polylysine complexes and by strategies that exploit receptor-mediated endocytosis for selective targeting (see, e.g., Curiel et al., Hum. Gene Then, 5:147-154, 1992; and Cristiano & Curiel, Cancer Gene Ther. 3:49-57, 1996). Non-viral methods for delivering polynucleotides are reviewed in Chang & Seymour, (Eds) Curr. Opin. Mol. Ther., vol. 2, 2000. These methods include contacting cells with naked DNA, cationic liposomes, or polypelexes of polynucleotides with cationic polymers and dendrimers for systemic administration (Chang & Seymour, Ibid.). Liposomes can be modified by incorporation
of ligands that recognize cell-surface receptors and allow targeting to specific receptors for uptake by receptor-mediated endocytosis (see, for example, Xu et al., *Mol. Genet. Metab.*, 64:193-197; 1998; and Xu et al., *Hum. Gene Ther.*, 10:2941-2952, 1999).

Tumor-targeting bacteria, such as *Salmonella*, are potentially useful for delivering genes to tumors following systemic administration (Low et al., *Nat. Biotechnol.* 17:37-41, 1999). Bacteria can be engineered *ex vivo* to penetrate and to deliver DNA with high efficiency into, for example, mammalian epithelial cells *in vivo* (see, e.g., Grillot-Courvalin et al., *Nat. Biotechnol.*.6:862-866, 1998). Degradation-stabilized oligonucleotides may be encapsulated into liposomes and delivered to patients by injection either intravenously or directly into a target site (for example, the origin of neuropathic pain). Alternatively, retroviral or adenoviral vectors, or naked DNA expressing anti-sense RNA for the inventive polypeptides, may be administered to patients. Suitable techniques for use in such methods are well known in the art.

The treatment compositions and methods of the present invention have been described, above, with respect to certain preferred embodiments. The Examples set forth below describe the results of specific experiments and are not intended to limit the invention in any fashion.

**EXAMPLE 1**

**Methyl 3-Aminosulfonvl-5-butvlammo-4-phenoxybenzoate**

(Bumetanide Methyl Ester)

To a slurry of bumetanide (1.2g, 3.29mmoi) in methanol (12mL) under nitrogen, was added a mixture of thionyl chloride (70uL) in methanol (6mL) over 5 minutes. After stirring for 5 minutes the reaction mixture became soluble. The reaction stirred for an additional 30 minutes, at which time the reaction was complete by thin layer chromatography (TLC). The methanol was removed under reduced pressure and the residue was brought up in ethyl acetate and washed with saturated sodium bicarbonate, water and brine. The ethyl acetate was dried over anhydrous magnesium sulfate and concentrated to a yield 1.1g (89%) of methyl 3-aminosulfonyl-5-butylamino-4-phenoxybenzoate as a white solid. Using similar methodology bumetanide ethyl ester, bumetanide isoamyl ester, bumetanide octyl ester and bumetanide benzyl ester, can be prepared.
EXAMPLE 2

3-Ammosulfonyl-5-butylamino-4-phenoxythiobenzoic Acid
(Thiobumetanide, Bumetanide -(C=OVSH Thioacid)

Bumetanide can be reacted thionyl chloride to make the corresponding acid chloride which can then be reacted with sodium hydrogen sulfide to give 3-aminosulfonyl-5-butylamino-4-phenoxythiobenzoic acid (thiobumetanide, S-bumetanide thioacid) by the methodology of Noble, P. and Tarbell, D. S., Ore. Synth., Coll. Vol. IV, John Wiley & Sons, Inc., New York, 1963, 924-927.

EXAMPLE 3

3-Aminosulfonyl-5-butylainino-4-phenoxythiobenzoic Acid
(Thiobumetanide, Bumetanide -(C=O)-SH Thioacid)

Bumetanide methyl ester can be reacted with hydrogen sulfide or sodium hydrogen sulfide to give, following acidification, 3-aminosulfonfl-5-butylamino-4-phenoxythiobenzoic acid (thiobumetanide, bumetanide thioacid).

EXAMPLE 4

Thiomethyl 3-Aminosulfonyl-5-butylamino-4-phenoxybenzoate
(Bumetanide S-Methyl Thioster)

In like manner to Example 1, bumetanide can be reacted with a catalytic amount of thionyl chloride in methanethiol (methyl mercaptan) to give thiomethyl 3-aminosulfonfl-5-butylamino-4-phenoxybenzoate. Using similar methodology with bumetanide and the corresponding thiols, bumetanide S-ethyl thioester, bumetanide S-isoamyl thioester, bumetanide S-octyl thioester and bumetanide S-benzyl thioester, can be prepared. Using similar methodology with dithiobumetanide and the corresponding alcohols, bumetanide O-ethyl thioester, bumetanide O-isoamyl thioester, bumetanide O-octyl thioester and bumetanide O-benzyl thioester, can be prepared.

EXAMPLE 5

3-Aminosulfonyl-S-butylamino-4-phenoxy-dithiobenzoic Acid
(Dithiobumetanide., Bumetanide -(C=S)-SH Dithioacid)

Thiobumetanide can be reacted thionyl chloride to make the corresponding thioacid chloride which can then be reacted with sodium hydrogen sulfide to give 3-aminosulfonfl-5-butylamino-4-phenoxy-dithiobenzoic acid (dithiobumetanide,
EXAMPLE 6

Methyl 3-Aminosulfonyl-5-butyIamino-4-phenoxy-dithiobenzoate

(Bumetanide Methyl Dithioester)

In like manner to Example 1, dithiobumetanide can be reacted with a catalytic amount of thionyl chloride in methanethiol (methyl mercaptan) to give methyl 3-aminosulfonyl-5-butyIamino-4-phenoxydithiobenzoate. Using similar methodology bumetanide ethyl dithioester, bumetanide isoamyl dithioester, bumetanide octyl dithioester and bumetanide benzyl dithioester, can be prepared.

EXAMPLE 7

Cyanomethyl 3-Aminosulfonyl-5-butyIamino-4-phenoxybenzoate

(Bumetanide Cyanomethyl Ester)

Bumetanide (1.0g, 2.7mmol) was dissolved in dimethylformamide (DMF) and chloroacetonitrile (195uL, 2.7mmol) was added followed by triethylamine (465uL). The reaction was heated to 100°C for 12 hours, TLC and liquid chromatography-coupled mass spectrometry (LC/MS) indicated the reaction was complete. The reaction was cooled to room temperature brought up in dichloromethane and washed with water, saturated ammonium chloride and reduced to a slurry. To the slurry was added water (25mL) and crude product precipitated as an off white solid. Pure cyanomethyl 3-aminosulfonyl-5-butyIamino-4-phenoxybenzoate (850mg) was obtained via recrystallization in acetonitrile. Using similar methodology bumetanide ethyl ester, bumetanide isoamyl ester, bumetanide octyl ester, and bumetanide benzyl ester, can be prepared.

EXAMPLE 8

Benzyl 3-Aminosulfonyl-5-butyIamino-4-phenoxybenzoate

(Bumetanide Benzyl Ester)

Bumetanide (1.15g, 3.15mmol) was dissolved in dimethylformamide (DMF, 10mL) and benzyl chloride (400uL, 2.8mmol) was added followed by triethylamine (480uL). The reaction was heated to 80°C for 12 hours, TLC and LC/MS indicated the reaction was complete. The reaction was cooled to room temperature brought up in dichloromethane and washed with water, saturated ammonium chloride and concentrated
to a thick slurry. To the slurry was added water (25mL), the resultant solids were filtered and dried in a vacuum oven at 50°C for 12 hours to yield 1.0g (80%) of benzyl 3-aminosulfonyl-5-butylamino-4-phenoxybenzoate.

**EXAMPLE 9**

*2-(4-Morpholino)ethyl 3-Aminosulfonyl-5-butylamino-4-phenoxybenzoate (Bumetanide Morpholinoethyl Ester)*

Bumetanide (1.2g, 3.29mmol) was dissolved in dimethylformamide (DMF, 12mL) and 4-(2-chloroethyl)morpholine hydrochloride (675mg, 3.62mmol) was added followed by triethylamine (1mL) and sodium iodide (500mg, 3.33mmol). The reaction was heated to 95°C for 8 hours, TLC and LC/MS indicated the reaction was complete. The reaction was cooled to room temperature brought up in dichloromethane and washed with water, saturated ammonium chloride and concentrated to dryness. After purification via biotage flash chromatography, the purified elute, on evaporation under vacuum, yielded 2-(4-morpholino)ethyl 3-aminosulfonyl-5-butylamino-4-phenoxybenzoate as a white solid (600mg, 62%).

**EXAMPLE 10**

*3-(N,N-Dimethylammonopropyl 3-Aminosulfonyl-5-butylamino-4-phenoxybenzoate (Bumetanide 3-(Dimethylaminopropyl) Ester)*

In similar manner to Example 54, bumetanide can be reacted with 3-(dimethylamino)propyl chloride hydrochloride, triethylamine and sodium iodide in dimethylformamide (DMF) to yield 3-(N,N-dimethylaminopropyl 3-aminosulfonyl-5-butylamino-4-phenoxybenzoate.

**EXAMPLE 11**

*3-(N,N-Dimethylaminopropyl 3-Aminosulfonyl-5-butylamino-4-phenoxy-dithiobenzoate (Bumetanide 3-(Dimethylaminopropyl) Dithioester)*

In similar manner to Example 10, dithiobumetanide can be reacted with 3-(dimethylamino)propyl chloride hydrochloride, triethylamine and sodium iodide in dimethylformamide (DMF) to yield 3-(N,N-dimethylaminopropyl 3-aminosulfonyl-5-butylamino-4-phenoxy-dithiobenzoate.
EXAMPLE 12

N,N-Diethylaminocarbonylmethyl 3-Aminosulfonyl-5-butylamino-4-phenoxybenzoate
(Bumetanide N,N-Diethylglycolamido Ester)

Bumetanide (1.2g, 3.29mmol) was dissolved in dimethylformamide (12mL) and
2-chloro-N,N-diethylacetamide (500mg, 3.35mmol) was added followed by triethylamine (0.68mL) and sodium iodide (500mg 3.33mmol). The reaction was heated to 95°C for 8 hours, TLC and LC/MS indicated the reaction was complete. The reaction was cooled to room temperature brought up in dichloromethane and washed with water, saturated ammonium chloride and reduced to a thick slurry. To the slurry was added water (25mL), the resultant solids precipitated from the solution. The product was filtered and dried in a vacuum oven at 50°C for 12 hours to yield 1.0g of N,N-diethylaminocarbonylmethyl 3-aminosulfonyl-5-butylamino-4-phenoxybenzoate.

EXAMPLE 13

N,N-Diethyl 3-Aminosulfonyl-5-buty lamino-4-phenoxybenzamide
(Bumetanide Diethylamide)

Bumetanide (1.16g, 3.2mmol) was dissolved in dichloromethane (10mL) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 690mg, 3.6mmol) was added and after 5 minutes N-hydroxybenzotriazole (HOBt, 498mg, 3.6mmol) was added and the solution was allowed to stir for an additional 5 minutes. Diethylamine (332uL, 3.2mmol) was added and the reaction was stirred for 2 hours. The reaction was washed with washed with saturated sodium bicarbonate, water, brine and dried with magnesium sulfate. The dichloromethane was removed under reduced pressure to yield 860mg (65%) of pure N,N-diethyl 3-aminosulfonyl-5-buty lamino-4-phenoxybenzamide.

EXAMPLE 14

N,N-Diethyl 3-Aminosulfonyl-5-buty lamino-4-phenoxythiobenzamide
(Bumetanide Diethylthioamide)

In similar manner to Example 5, dithiobumetanide can be reacted with thionyl chloride to give the thioacid chloride, which can be reacted with diethylamine to afford N,N-diethyl 3-aminosulfonyl-5-buty lamino-4-phenoxythiobenzamide.
EXAMPLE 15
N,N-DibenzyI 3-Aminosulfonyl-5-butyIamino-4-phenoxybenzamide
(Bumetanide Dibenzylamide)
Bumetanide (960mg, 2.6mmol) was dissolved in dimethylformamide (DMF 5 10mL) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 560mg, 3.6mmol) was added and after 10 minutes 1-hydroxybenzotriazole (HOBt, 392mg, 2.9mmol) was added and the solution was allowed to stir for an additional 10 minutes. Dibenzylamine (1mL, 5.2mmol) was added and the reaction was stirred for 2 hours, at which time the reaction was complete by LC/MS. The reaction was poured into saturated ammonium chloride (2OmL) and extracted with ethyl acetate (2x10OmL). The ethyl acetate was washed with saturated sodium bicarbonate, water, brine and dried over anhydrous magnesium sulfate. The ethyl acetate was removed under reduced pressure to yield 10g (75%) of N,N-dibenzyl 3-aminosulfonyl-5-butyIamino-4-phenoxybenzamide as white solid.

EXAMPLE 16
Benzyltrimethylammonium 3-Aminosulfonyl-5-butyIamino-4-phenoxybenzoate
(Bumetanide Benzyltrimethylammonium Salt)
To a solution of benzyltrimethylammonium hydroxide (451mg, 2.7mmol) in water (10mL) was added bumetanide (1g, 2.7 mmol) over a period of 5 minutes. The reaction mixture became clear after 10 minutes of stirring. The water was removed under reduced pressure to yield a crude colorless oil. Pure product was obtained from recrystallization of the oil with water and heptane to yield 690mg of benzyltrimethylammonium 3-aminosulfonyl-5-butyIamino-4-phenoxybenzoate as light pink crystals.

EXAMPLE 17
Cetyltrimethylammonium 3-Aminosulfonyl-5-butyIamino-4-phenoxybenzoate
(Bumetanide Cetyltrimethylammonium Salt)
In similar manner to Example 16, bumetanide can be reacted with cetyltrimethylammonium hydroxide in water to yield cetyltrimethylammonium 3-aminosulfonyl-5-butyIamino-4-phenoxybenzoate.
EXAMPLE 18

N,N-Pimethylaminocarbonylmethyl 3-Aminosulfonyl-5-butylamino-4-phenoxybenzoate
(Bumetanide N,N-Dimethylglycolamido Ester)

Bumetanide (1.2g, 3.29mmol) was dissolved in dimethylformamide (DMF, 10mL) and 2-chloro-N,N-dimethylacetamide (410uL, 3.9mmol) was added followed by triethylamine (0.7OmL) and sodium iodide (545mg, 3.6mmol). The reaction was heated to 50°C for 10 hours, TLC and LC/MS indicated the reaction was complete. The solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate and washed with saturated sodium bicarbonate, water, and brine and dried over anhydrous magnesium sulfate. The ethyl acetate was removed under reduced pressure and the product was purified via flash chromatography to yield 685mg (60%) of pure N,N-dimethylaminocarbonylmethyl 3-aminosulfonyl-5-butylamino-4-phenoxybenzoate.

EXAMPLE 19

t-ButylcarbonyloxymethylS-Aminosttlfonyl-S-butylamino^-phenoxybenzoate
(Bumetanide Pivaxetil Ester)

Bumetanide (1.2g, 3.29mmol) was dissolved in dimethylformamide (DMF, 10mL) and chloromethyl pivalate (575uL, 3.9mmol) was added followed by triethylamine (0.7OmL) and sodium iodide (545mg, 3.6mmol). The reaction was heated to 50°C for 10 hours, TLC and LC/MS indicated the reaction was complete. The solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate and washed with saturated sodium bicarbonate, water, and brine and dried over anhydrous magnesium sulfate. The ethyl acetate was removed under reduced pressure and the product was purified via flash chromatography to yield 653mg (60%) of pure t-butylcarbonyloxymethyl 3-aminosulfonyl-5-butylamino-4-phenoxybenzoate.

EXAMPLE 20

t-Butylcarbonyloxymethyl 3-Aminosulfonyl-5-butylamino-4-phenoxy-dithiobenzoate
(Bumetanide Pivaxetil Dithioester)

In similar manner to Example 19, dithiobumetanide can be reacted with chloromethyl pivalate, triethylamine and sodium iodide in dimethylformamide (DMF) to yield t-butylcarbonyloxymethyl 3-aminosulfonyl-5-butylamino-4-phenoxy-dithiobenzoate.
EXAMPLE 21

Ethylcarbonyloxymethyl 3-Aminosulfonfyl-5-butylammono-4-phenoxybenzoate
(Bumetanide Propaxetil Ester)

In similar manner to Example 19, bumetanide can be reacted with chloromethyl propionate, triethylamine and sodium iodide in dimethylformamide (DMF) to yield ethylcarbonyloxymethyl 3-aminosulfonfyl-5-butylamino-4-phenoxybenzoate.

EXAMPLE 22

Ethylcarbonyloxymethyl 3-Aminosulfonfyl-5-butylamino-4-phenoxy-dithiobenzoate
(Bumetanide Propaxetil Dithioester)

In similar manner to Example 21, dithiobumetanide can be reacted with chloromethyl propionate, triethylamine and sodium iodide in dimethylformamide (DMF) to yield ethylcarbonyloxymethyl 3-aminosulfonfyl-5-butylamino-4-phenoxy-dithiobenzoate.

EXAMPLE 23

Methyl 3-Aminosulfonfyl-4-phenoxy-5-(1-pyrrolidinyl)benzoate
(Piretanide Methyl Ester)

In similar manner to Example 1, piretanide can be reacted with thionyl chloride and methanol to yield methyl 3-aminosulfonfyl-4-phenoxy-5-(1-pyrrolidinyl)benzoate. Using similar methodology piretanide ethyl ester, piretanide isoamyl ester, piretanide octyl ester and piretanide benzyl ester can be prepared.

EXAMPLE 24

3-Aminosthifonyl-4-phenoxy-5-(1-pyrrolidinyl)-thiobenzoic Acid
(Thiopiretanide, Piretanide -(C=OVSH Thioacid)

Piretanide can be reacted thionyl chloride to make the corresponding acid chloride which can then be reacted with sodium hydrogen sulfide to give 3-aminosulfonfyl-4-phenoxy-5-(1-pyrrolidinyl)-thiobenzoic acid (thiopiretanide, S-piretanide thioacid) by the methodology of Noble, P. and Tarbell, D. S., Org. Synth., Coll. Vol. IV, John Wiley & Sons, Inc., New York, 1963, 924-927.
EXAMPLE 25

3-Aminosulfonfyl-4-phenoxy-5-(l-pyrrolidinyl)-thiobenzoic Acid
(Thiopiretanide, Piretanide -(C=Q)-SH Thioacid)

Piretanide methyl ester can be reacted with hydrogen sulfide or sodium hydrogen sulfide to give 3-aminosulfonfyl-4-phenoxy-5-(l-pyrrolidinyl)-thiobenzoic acid (thiopiretanide, S-piretanide thioacid).

EXAMPLE 26

Thiomethyl 3-aminosulfonfyl-4-phenoxy-5-(l-pyrrolidinyl)benzoate
(Piretanide S-Methyl Thioester)

In like manner to Example 1, piretanide can be reacted with a catalytic amount of thionyl chloride in methanethiol (methyl mercaptan) to give thiomethyl 3-aminosulfonfyl-4-phenoxy-5-(l-pyrrolidinyl)benzoate. Using similar methodology with piretanide and the corresponding thiols, piretanide S-ethyl thioester, piretanide S-isoamyl thioester, piretanide S-octyl thioester and piretanide S-benzyl thioester, can be prepared. Using similar methodology with dithiopiretanide and the corresponding alcohols, piretanide O-ethyl thioester, piretanide O-isoamyl thioester, piretanide O-octyl thioester and piretanide O-benzyl thioester, can be prepared.

EXAMPLE 27

3-Aminosulfonfyl-4-phenoxy-5-(l-pyrrolidinyl)-dithiobenzoic Acid
(Dithiopiretanide, Piretanide -(C=VSVSH Dithioacid)

Thiopiretanide can be reacted thionyl chloride to make the corresponding thioacid chloride which can then be reacted with sodium hydrogen sulfide to give 3-aminosulfonfyl-4-phenoxy-5-(1-pyrrolidinyl)-dithiobenzoic acid (dithiopiretanide, piretanide dithioacid) by the methodology of Noble, P. and Tarbell, D. S., Org. Synth., Coll. Vol. IV, John Wiley & Sons, Inc., New York, 1963, 924-927.

EXAMPLE 28

Methyl 3-aminosulfonfyl-4-phenoxy-5-(l-pyrrolidinyl)-dithiobenzoate
(Piretanide Methyl Dithioester)

In like manner to Example 1, dithiopiretanide can be reacted with a catalytic amount of thionyl chloride in methanethiol (methyl mercaptan) to give methyl 3-amnosulfonfyl-4-phenoxy-5-(l-pyrrolidinyl)-dithiobenzoate. Using similar methodology
piretanide ethyl dithioester, piretanide isoamyl dithioester, piretanide octyl dithioester and piretanide benzyl dithioester can be prepared.

**EXAMPLE 29**

**Cyanomethyl 3-Aminosulfonyl-4-phenoxy-5-(1-pyrrolidinyl)benzoate**

(Piretanide Cyanomethyl Ester)

In similar manner to Example 7, piretanide can be reacted with chloroacetonitrile and triethylamine in DMF to yield cyanomethyl 3-aminosulfonyl-4-phenoxy-5-(1-pyrrolidinyl)benzoate.

**EXAMPLE 30**

**Benzyl 3-Aminosulfonyl-4-phenoxy-5-(1-pyrrolidinyl)benzoate**

(Piretanide Benzyl Ester)

In similar manner to Example 8, piretanide can be reacted with benzyl chloride and triethylamine in DMF to yield benzyl 3-aminosulfonyl-4-phenoxy-5-(1-pyrrolidinyl)benzoate.

**EXAMPLE 31**

**2-(4-Morpholino)ethyl 3-Aminosulfonyl-4-phenoxy-5-(1-pyrrolidinyl)benzoate**

(Piretanide Morpholinoethyl Ester)

In similar manner to Example 9, piretanide can be reacted with 4-(2-chloroethyl)morpholine hydrochloride, triethylamine and sodium iodide in DMF to yield 2-(4-morpholino)ethyl 3-aminosulfonyl-4-phenoxy-5-(1-pyrrolidinyl)benzoate.

**EXAMPLE 32**

**3-(N,N-Dimethylaminopropyl) 3-Aminosulfonyl-4-phenoxy-5-(1-pyrrolidinyl)benzoate**

(Piretanide 3-(Dimethylaminopropyl) Ester)

In similar manner to Example 54, piretanide can be reacted with 3-(dimethylamino)propyl chloride hydrochloride, triethylamine and sodium iodide in dimethylformamide (DMF) to yield 3-(N,N-dimethylaminopropyl) 3-aminosulfonyl-4-phenoxy-5-(1-pyrrolidinyl)benzoate.
EXAMPLE 33

\[
3-(N,N\text{-Dimethylaminopropyl} \ 3\text{-Aminosulfonyl-4-phenoxy-5-(1-pyrrolidinyl)dithiobenzoate)}
\]

[Piretanide 3-(Dimethylaminopropyl) Dithioester]

In similar manner to Example 32, dithiopiretanide can be reacted with 3-(dimethylamino)propyl chloride hydrochloride, triethylamine and sodium iodide in dimethylformamide (DMF) to yield 3-(N,N-dimethylaminopropyl 3-aminosulfanyl-4-phenoxy-5-(1-pyrrolidinyl)dithiobenzoate.

EXAMPLE 34

\[
N,N\text{-Diethylaminocarbonylmethyl} \ 3\text{-Aminosulfanyl-4-phenoxy-5-(1-pyrrolidinyl)benzoate)}
\]

(Piretanide N,N-Diethylglycolamide Ester)

In similar manner to Example 12, piretanide can be reacted with 2-chloro-N,N-diethylacetamide, triethylamine and sodium iodide in dimethylformamide (DMF) to yield N,N-diethylaminocarbonylmethyl 3-aminosulfanyl-4-phenoxy-5-(1-pyrrolidinyl)benzoate.

EXAMPLE 35

\[
N,N\text{-Diethyl} \ 3\text{-Aminosulfanyl-4-phenoxy-5-fl-pyrrolidinyl)}\text{benzoate)}
\]

(Piretanide Diethylamide)

In similar manner to Example 13, piretanide can be reacted with EDC, HOBt and diethylamine in DMF to yield N,N-diethyl 3-aminosulfanyl-4-phenoxy-5-(1-pyrrolidinyl)benzamide.

EXAMPLE 36

\[
N,N\text{-Diethyl} \ 3\text{-Aminosulfanyl-4-phenoxy-5-fl-pyrrolidinyl)}\text{benzoate)}
\]

(Piretanide Diethylthioamide)

In similar manner to Example 35, dithiopiretanide can be reacted with EDC, HOBt and diethylamine in DMF to yield N,N-diethyl 3-aminosulfanyl-4-phenoxy-5-(1-pyrrolidinyl)thiobenzamide.
EXAMPLE 37

NJX-DibenzyI 3-AminosuIfonyI-4-phenoxy-5-(1-pyrrolidinyl)benzoate
(Piretanide Dibenzylamide)

In similar manner to Example 15, piretanide can be reacted with EDC, HOBt and
dibenzyllamine in DMF to yield N,N-dibenzy 3-aminosulfonyl-4-phenoxy-5-(1-
pyrrolidinyl)benzamide.

EXAMPLE 38

Benzyltrimethylammonium 3-Aminosulfonyl-4-phenoxy-5-(1-pyrrolidinyl)benzoate
(Piretanide Benzyltrimethylammonium Salt)

In similar manner to Example 16, piretanide can be reacted with
benzyltrimethylammonium hydroxide to yield benzyltrimethylammonium 3-
aminosulfonyl-4-phenoxy-5-(1-pyrrolidinyl)benzoate.

EXAMPLE 39

Cetyltrimethylammonium 3-Aminosulfonyl-4-phenoxy-5-(1-pyrrolidinyl)benzoate
(Piretanide Cetyltrimethylammonium Salt)

In similar manner to Example 17, piretanide can be reacted with
cetyltrimethylammonium hydroxide in water to yield cetyltrimethylammonium 3-
aminosulfonyl-4-phenoxy-5-(1-pyrrolidinyl)benzoate.

EXAMPLE 40

N,N-Dimethylaminocarbonvlmethyl 3-Aminosulfonyl-4-phenoxy-5-(1-
pyrrolidinyl)benzoate
(Piretanide N,N-Pimethylglycolamido Ester)

In similar manner to Example 18, piretanide can be reacted with 2-chloro-N,N
dimethylacetamide, triethylamine and sodium iodide in DMF to yield N,N-
dimethylaminocarbonyl methyl 3-aminosulfonyl-4-phenoxy-5-(1-pyrrolidinyl)benzoate.

EXAMPLE 41

t-Butvlcarbonyloxymethyl 3-Aminosulfonyl-4-phenoxy-5-(1-pyrrolidinyl)benzoate
(Piretanide Pivaxetil Ester)

In similar manner to Example 19, piretanide can be reacted with chloromethyl
pivalate, triethylamine and sodium iodide in DMF to yield t-buty1carbonyloxymethyl 3-
aminosulfonyl-4-phenoxy-5-(1-pyrrolidinyl)benzoate.
EXAMPLE 42

t-Butylcarbonyloxymethyl 3-Aminosulfonyl-4-phenoxy-5-(1-pyrrolidinyl)dithiobenzoate
(Piretanide Pivacetil Pithioester)

In similar manner to Example 41, dithiopiretanide can be reacted with chloromethyl pivalate, triethylamine and sodium iodide in DMF to yield t-butylcarbonyloxymethyl 3-aminosulfonyl-4-phenoxy-5-(1-pyrrolidinyl)dithiobenzoate.

EXAMPLE 43

Ethylcarbonyloxymethyl 3-Aminosulfonfyl-4-phenoxy-5-fl-pyrrolidinyl)benzoate
(Piretanide Propaxetil Ester)

In similar manner to Example 21, piretanide can be reacted with chloromethyl propionate, triethylamine and sodium iodide in DMF to yield ethylcarbonyloxymethyl 3-aminosulfonyl-4-phenoxy-5-((1-pyrrolidinyl)benzoate.

EXAMPLE 44

Ethyl S-Aminosulfonyl-chloro-1-flCl-fluranymethyDaminolbenzoate (Furosemide Ethyl Ester)

The method of Bundgaard, H., Norgaard, T. and Nielsen, N. M., Int. J. Pharmaceutics, 1988, 42, 217-224, can be employed to prepare ethyl 5-aminosulfonyl-4-chloro-2-[(2-furanylmethyl)amino]benzoate, m.p. 163 — 165°. Using similar methodology furosemide methyl ester, furosemide isoamyl ester, furosemide octyl ester and furosemide benzyl ester can be prepared.

EXAMPLE 45

Methyl 5-Aminosulfonfl-4-chloro-2-f(2-furanyimethyl)aminolbenzoate (Furosemide Methyl Ester)


EXAMPLE 46

5-Aminosulfonyl-4-chloro-2-f(2-furanylmethyl)aminolthiobenzoic Acid
(Thiofurosemide, Furosemide -(C=O)-SH Thioacid)

Furosemide can be reacted thionyl chloride to make the corresponding acid chloride which can then be reacted with sodium hydrogen sulfide to give 5-aminosulfonyl-4-chloro-2-[(2-furanylmethyl)amino]thiobenzoic acid (thiofurosemide, S-
furosemide thioacid) by the methodology of Noble, P. and Tarbell, D. S., Org. Synth.,

EXAMPLE 47

5-Aminosulfonyl-4-chloro-2-f(2-furanylmethyl)aminothiobenzoic Acid
(Thiofurosemide, Furosemide -(C=O)-SH Thioacid)

Furosemide methyl ester can be reacted with hydrogen sulfide or sodium hydrogen sulfide to give, following acidification, 3-aminosulfonyl-5-butylamino-4-phenoxythiobenzoic acid (thiofurosemide, S-furosemide thioacid).

EXAMPLE 48

Thiomethyl 5-Aminosulfonyl-4-chloro-2-f(2-furanylmethyl)aminothiobenzoate
(Furosemide S-Methyl Thioester)

In like manner to Example 1, bumetanide can be reacted with a catalytic amount of thionyl chloride in methanethiol (methyl mercaptan) to give thiomethyl 5-aminosulfonyl-4-chloro-2-f(2-furanylmethyl)aminothiobenzoate. Using similar methodology with furosemide and the corresponding thiols, furosemide S-ethyl thioester, furosemide S-isoamyl thioester, furosemide S-octyl thioester and furosemide S-benzyl thioester, can be prepared. Using similar methodology with dithio furosemide and the corresponding alcohols, furosemide O-ethyl thioester, furosemide O-isoamyl thioester, furosemide O-octyl thioester and furosemide O-benzyl thioester, can be prepared.

EXAMPLE 49

5-Aminosulfonyl-4-chloro-2-f(2-furanylmethyl)amino1-dithiobenzoic Acid
(Dithiofurosemide, Furosemide -(C=S)-SH Dithioacid)

Thiofurosemide can be reacted thionyl chloride to make the corresponding thioacid chloride which can then be reacted with sodium hydrogen sulfide to give 5-aminosulfonyl-4-chloro-2-f(2-furanylmethyl)amino1-dithiobenzoic acid (dithiofurosemide, furosemide dithioacid) by the methodology of Noble, P. and Tarbell, D. S., Org. Synth., Coll. Vol. IV, John Wiley & Sons, Inc., New York, 1963, 924-927.
EXAMPLE 50

Methyl 5-Aminosulfonyl-4-chloro-2-furanylmethylamino)dithiobenzoate
(Furosemide Methyl Dithioester)

In like manner to Example 1, dithiofurosemide can be reacted with a catalytic amount of tmonyl chloride in methanethiol (methyl mercaptan) to give methyl 5-aminosulfonyl-4-chloro-2-[(2-furanylmethyl)amino)dithiobenzoate. Using similar methodology furosemide ethyl dithioester, furosemide Soamyl dithioester, furosemide octyl dithioester and furosemide benzyl dithioester can be prepared.

EXAMPLE 51

Cyanomethyl 5-Aminosulfonyl-4-chloro-2-[(2-furanylmethyl)aminolbenzoate
(Furosemide Cyanomethyl Ester)

In similar manner to Example 7, furosemide can be reacted with chloroacetonitrile and triethylamine in DMF to yield cyanomethyl 5-aminosulfonyl-4-chloro-2-[(2-furanylmethyl)amino]benzoate.

EXAMPLE 52

Benzyl 5-Aminosulfonyl-4-chloro-2-[(2-furanylmethyl)amino]benzoate (Furosemide Benzyl Ester)

In similar manner to Example 8, furosemide can be reacted with benzyl chloride and triethylamine in DMF to yield benzyl 5-aminosulfonyl-4-chloro-2-[(2-furanylmethyl)amino]benzoate.

EXAMPLE 53

2-(4-Morpholino)ethyl 5-Aminosulfonyl-4-chloro-2-furanylmethyldaminobenzoate
(Furosemide Morpholinoethyl Ester)

EXAMPLE 54

3-(N,N-Dimethylaminopropyl 5-Aminosulfonyl-4-chloro-2-[(2-furanyl)methyl]amino]benzoate
(Furosemide 3-(Dimethylaminopropyl) Ester)


EXAMPLE 55

3-(N,N-Dimethylaminopropyl 5-Aminosulfonyl-4-chloro-2-[(2-furanyl)methyl]amino]dithiobenzoate
(Furosemide 3-(Dimethylaminopropyl) Dithioester)

In similar manner to Example 54, dithiofurosemide can be reacted with 3-(dimethylamino)propyl chloride hydrochloride, triethylamine and sodium iodide in dimethylformamide (DMF) to yield 3-(N,N-dimethylaminopropyl 5-aminosulfonyl-4-chloro-2-[(2-furanyl)methyl]amino]dithiobenzoate.

EXAMPLE 56

N,N-Diethylaminocarbonylmethyl 5-Aminosulfonyl-4-chloro-2-[(2-furanyl)methyl]amino]benzoate
(Furosemide N,N-Diethylglycolamido Ester)


EXAMPLE 57

N,N-Diethyl 5-Aminosulfonyl-4-chloro-2-[(2-furanyl)methyl]amino]benzamide
(Furosemide Diethylamide)

In similar manner to Example 13, furosemide can be reacted with EDC, HOBt and diethylamine in DMF to yield N,N-diethyl 5-aminosulfonyl-4-chloro-2-[(2-furanyl)methyl]amino]benzamide.
EXAMPLE 58

N,N-Diethyl 5-Aminosulfonyl-4-chloro-2-f(2-furanylmethyl)amino]benzamide
(Furosemide Piethylthioamide)

In similar manner to Example 57, dithiofurosemide can be reacted with EDC, HOBt and diethylamine in DMF to yield N,N-diethyl 5-aminosulfonyl-4-chloro-2-[2-furanylmethyl]amino]thiobenzamide.

EXAMPLE 59

N,N-Dibenzyl 5-Aminosulfonyl-4-chloro-2-f(2-furanylmethyl)amino]benzamide
(Furosemide Dibenzylamide)

In similar manner to Example 15, furosemide can be reacted with EDC, HOBt and dibenzylamine in DMF to yield N,N-dibenzyl 5-aminosulfonyl-4-chloro-2-[2-furanylmethyl]amino]benzamide.

EXAMPLE 60

Benzyltrimethyl ammonium 5-Aminosulfonyl-4-chloro-2-f(2-furanylmethyl)amino]benzoate
(Furosemide Benzyltrimethyl ammonium Salt)

In similar manner to Example 16, furosemide can be reacted with benzyltrimethyl ammonium hydroxide to yield benzyltrimethyl ammonium 5-aminosulfonyl-4-chloro-2-[2-furanylmethyl]amino]benzoate.

EXAMPLE 61

Cetyltrimethyl ammonium 5-Aminosulfonyl-4-chloro-2-f(2-furanylmethyl)amino]benzoate
(Furosemide Cetyltrimethyl ammonium Salt)

In similar manner to Example 17, furosemide can be reacted with cetyltrimethyl ammonium hydroxide in water to yield cetyltrimethyl ammonium 5-aminosulfonyl-4-chloro-2-[2-furanylmethyl]amino]benzoate.

EXAMPLE 62

N,N-Dimethylaminocarbonylmethyl 5-Aminosulfonyl-4-chloro-2-f(2-furanylmethyl)amino]benzoate
(Furosemide N,N-Dimethylglycolamido Ester)

The method of Bundgaard, H., Norgaard, T. and Nielsen, N. M., Int. J. Pharmaceutics, 1988, 42, 217-224, can be employed to prepare N,N-

**EXAMPLE 63**

\[
\text{t-Butylcarbonyloxymethyl 5-Aminosulfonyl-4-chloro-2-f(2-furanylmethyl)amino}1\text{benzoate}
\]

(Furosemide Pivaxetil Ester)


**EXAMPLE 64**

\[
\text{t-Butylcarbonyloxymethyl 5-Aminosulfonyl-4-chloro-2-f(2-furanylmethyl)amino}1\text{dithiobenzoate}
\]

(Furosemide Pivaxetil Dithioester)

In similar manner to Example 63, dithiofurosemide can be reacted with chloromethyl pivalate, triethylamine and sodium iodide in dimethylformamide (DMF) to yield t-butylcarbonyloxymethyl 5-aminosulfonyl-4-chloro-2-[(2-furanylmethyl)amino]dithiobenzoate.

**EXAMPLE 65**

\[
\text{Ethylcarbonyloxymethyl 5-Aminosulfonyl-4-chloro-2-f(2-}
\]

\[
\text{furanylmethyl)amino}1\text{benzoate}
\]

(Furosemide Propaxetil Ester)


**EXAMPLE 66**

\[
\text{5-ri-(t-Butylcarbonyloxymethyl)-lH-tetrazol-5-vn-2-chloro-4-f(2-}
\]

\[
\text{thienylmethyDaminolbenzenesulfonamide}
\]

(Tetrazolyl-Substituted Azosemide)

In similar manner to Example 19, azosemide can be reacted with chloromethyl pivalate, triethylamine and sodium iodide in DMF to yield 5-[l-(t-

EXAMPLE 67

2-Chloro-5-(ethylcarbonyloxymethyl)-1H-tetrazol-5-yl-4-(2-thienylmethyl)amino]benzenesulfonamide
(Tetrazolyl-Substituted Azosemide)

In similar manner to Example 19, azosemide can be reacted with chloromethyl propionate, triethylamine and sodium iodide in DMF to yield 2-chloro-5-[1-(ethylcarbonyloxymethyl)-1H-tetrazol-5-yl]-4-[2-thienylmethyl)amino]benzenesulfonamide.

EXAMPLE 68

2-Chloro-5-(hydroxymethyl)-1H-tetrazol-5-yl-4-[2-thienylmethyl)amino]benzenesulfonamide
(Tetrazolyl-Substituted Azosemide)

Azosemide can be reacted with formaldehyde in methylene chloride, methylene chloride-DMF mixtures or DMF to yield 2-chloro-5-[1-(hydroxymethyl)-1H-tetrazol-5-yl]-4-[2-thienylmethyl)amino]benzenesulfonamide.

EXAMPLE 69

2-Chloro-5-fl-(methoxymethyl)-1H-tetrazol-5-yl]-4-[2-thienylmethylPamino]benzenesulfonamide
(Tetrazolyl-Substituted Azosemide)

Azosemide can be reacted with formaldehyde, methanol and a strong acid in methylene chloride, methylene chloride-DMF mixtures or DMF to yield 2-chloro-5-[1-(methoxymethyl)-1H-tetrazol-5-yl]-4-[2-thienylmethyl)amino]benzenesulfonamide.

EXAMPLE 70

2-Chloro-5-n-(methylthiomethyln-H-tetrazol-5-yn-4-[2-thienylmethyl)amino]benzenesulfonamide
(Tetrazolyl-Substituted Azosemide)

Azosemide can be reacted with formaldehyde, methanethiol and a strong acid in methylene chloride, methylene chloride-DMF mixtures or DMF to yield 2-chloro-5-[1-(methylthiomethyl)-1H-tetrazol-5-yl]-4-[2-thienylmethyl)amino]benzenesulfonamide.
EXAMPLE 71

5-fl-(Benzyloxymethyl)-1H-tetrazol-5-yl-2-chloro-4-[(2-thienylmethyl)amino]benzenesulfonamide
(Tetrazoly1-Substituted Azosemide)

Azosemide can be reacted with benzyl chloromethyl ether, triethylamine and sodium iodide in DMF to yield 5-[1-(benzyloxymethyl)-1H-tetrazol-5-yl]-2-chloro-4-[(2-thienylmethyl)amino]benzenesulfonamide.

EXAMPLE 72

Benzyltrimethylammonium Salt of 2-Chloro-5-(1H-tetrazol-5-yl)-4-[(2-thienylmethyl)amino]benzenesulfonamide
(Azosemide Benzyltrimethylammonium Salt)

In similar manner to Example 16, azosemide can be reacted with benzyltrimethylammonium hydroxide in water to yield the benzyltrimethylammonium salt of 2-chloro-5-(1H-tetrazol-5-yl)-4-[(2-thienylmethyl)amino]benzenesulfonamide.

EXAMPLE 73

Cetyltrimethylammonium Salt of 2-Chloro-5-(1H-tetrazol-5-yl)-4-(2-thienylmethyl)aminobenzenesulfonamide
(Azosemide Cetyltrimethylammonium Salt)

In similar manner to Example 16, azosemide can be reacted with cetyltrimethylammonium hydroxide in water to yield the cetyltrimethylammonium salt of 2-chloro-5-(1H-tetrazol-5-yl)-4-[(2-thienylmethyl)amino]benzenesulfonamide.

EXAMPLE 74

3-Isopropylcarbamylsulfonamido-4-(3’-methylphenyl)aminopyridinium t-Butylcarbonyloxymethochloride
(Pyridinium-Substituted Torsemide Salt)

In similar manner to Example 19, torsemide can be reacted with chloromethyl pivalate, triethylamine and sodium iodide in DMF to yield 3-isopropylcarbamylsulfonamido-4-(3’-methylphenyl)aminopyridinium t-butylcarbonyloxymethochloride and some 3-isopropylcarbamylsulfonamido-4-(3’-nøethy1phenyl)aminopyridinium t-butylcarbonyloxymethioiodide.
EXAMPLE 75
3-Isopropylcarbamylsulfonamido-4-(3'-methylphenyl)aminopyridinium
Ethylcarbonyloxymethochloride
(Pyridinium-Substituted Torsemide Salt)
In a similar manner to Example 19, torsemide can be reacted with chloromethyl propionate, triethylamine and sodium iodide in DMF to yield 3-isopropylcarbamylsulfonamido-4-(3'-methylphenyl)aminopyridinium ethylcarbonyloxymethochloride and some 3-isopropylcarbamylsulfonamido-4-(3'-methylphenyl)aminopyridinium ethylcarbonyloxymethiodide.

EXAMPLE 76
3-Isopropylcarbamylsulfonamido-4-(3'-methylphenyl)aminopyridinium
benzyloxymethochloride
(Pyridinium-Substituted Torsemide Salt)
In a similar manner to Example 8, torsemide can be reacted with benzyl chloromethyl ether and triethylamine in DMF to yield 3-isopropylcarbamylsulfonamido-4-(3'-methylphenyl)aminopyridinium benzyloxymethochloride.

EXAMPLE 77
3-Isopropylcarbamylsulfonamido-4-(3'-methylphenyl)aminopyridinium
methoxymethochloride
(Pyridinium-Substituted Torsemide Salt)
In a similar manner to Example 8, torsemide can be reacted with methyl chloromethyl ether and triethylamine and in DMF to yield 3-isopropylcarbamylsulfonamido-4-(3'-methylphenyl)aminopyridinium methoxymethochloride.

EXAMPLE 78
S-Isopropylcarbamylsulfonamido^-O'-methylphenyldaminopyridinium
phenylmethochloride
(Pyridinium-Substituted Torsemide Salt)
In a similar manner to Example 8, torsemide can be reacted with benzyl chloride and triethylamine in DMF to yield 3-isopropylcarbamylsulfonamido-4-(3'-methylphenyl)aminopyridinium phenylmethochloride.
EXAMPLE 79

3-Isopropylcarbamysulfonamido-4-(3’-methylphenyl)aminopyridinium
Benzylthiomethochloride
(Pyridinium-Substituted Torsemide Salt)

In a similar manner to Example 8, torsemide can be reacted with benzyl chloromethyl thioether and triethylamine in DMF to yield 3-isopropylcarbamysulfonamido-4-(3’-methylphenyl)aminopyridinium benzylthiomethochloride.

EXAMPLE 80

3-Isopropylcarbamysulfonamido-4-(3’-methylphenyl)aminopyridinium
Methylthiomethochloride
(Pyridinium-Substituted Torsemide Salt)

In a similar manner to Example 8, torsemide can be reacted with methyl chloromethyl thioether and triethylamine in DMF to yield 3-isopropylcarbamysulfonamido-4-(3’-methylphenyl)aminopyridinium methylthiomethochloride.

EXAMPLE 81

Methoxy(polyethyleneoxy) n-ethyl 3-Aminosulfonyl-5-butylamino-4-
phenoxybenzoate (Bumetanide mPEG350 Esters)

In a manner similar to Example 8, bumetanide can be reacted with MeO-PEG350-C1 (Biolink Life Sciences, Inc., Cary, NC, BLS-106-350) and triethylamine in DMF to yield methoxy(polyethyleneoxy) n-1-ethyl 3-aminosulfonyl-5-butylamino-4-phenoxybenzoate where n is in the 7-8 range.

EXAMPLE 82

Methoxy(polyethyleneoxy)n-ethyl 3-Aminosulfonyl-5-butylamino-4-
phenoxybenzoate (S-Bumetanide mPEG350 Thioesters)

In a manner similar to Example 8, thiobumetanide can be reacted with MeO-PEG350-C1 (Biolink Life Sciences, Inc., Cary, NC, BLS-106-350) and triethylamine in DMF to yield methoxy(polyethyleneoxy)n-1-ethyl 3-aminosulfonyl-5-butylamino-4-phenoxy-thiobenzoate where n is in the 7-8 range.
EXAMPLE 5
Methoxy(polyethyleneoxy)$_n$-i-ethyl 3-Aminosulfonyl-5-butylamino-4-
phenoxybenzoate (Bumetanide mPEG1000 Esters)

In a manner similar to Example 8, bumetanide can be reacted with MeO-
PEG1000-OTs (Biolink Life Sciences, Inc., Cary, NC, BLS-107-1000) and triethylamine
in DMF to yield methoxy(polyethyleneoxy)$_n$-i-ethyl 3-aminosulfonyl-5-butylamino-4-
phenoxybenzoate where n is in the 19-24 range. In similar manner S-bumetanide
mPEG1000 thiosters can be formed with S-thiobumetanide, MeO-PEG1000-OTs (Biolink
Life Sciences, Inc., Cary, NC, BLS-107-1000) and triethylamine in DMF.

EXAMPLE 84
Methoxy(polyethyleneoxy)$_n$-i-ethyl 3-Aminosulfonyl-5-butylamino-4-
phenoxybenzoate (Bumetanide mPEG1000 Dithioesters)

In a manner similar to Example 8, dithiobumetanide can be reacted with MeO-
PEG1000-OTs (Biolink Life Sciences, Inc., Cary, NC, BLS-107-1000) and triethylamine
in DMF to yield methoxy(polyethyleneoxy)$_n$-i-ethyl 3-aminosulfonyl-5-butylamino-4-
phenoxy-dithiobenzoate where n is in the 19-24 range.

EXAMPLE 85
Methoxy(polyethyleneoxy)$_n$-i-ethyl 3-Aminosulfonyl-4-phenoxy-5-(l-
pyrrolidinyl)benzoate (Piretanide mPEG350 Esters)

In similar manner to Example 8, piretanide can be reacted with MeO-PEG350-Cl
(Biolink Life Sciences, Inc., Cary, NC, BLS-106-350) and triethylamine in DMF to yield
methoxy(polyethyleneoxy)$_n$-i-ethyl 3-aminosulfonyl-4-phenoxy-5-(l-
pyrrolidinyl)benzoate where n is in the 7-8 range. In similar manner bumetanide
mPEG350 dithioesters can be formed with dithiobumetanide, MeO-PEG350-Cl (Biolink
Life Sciences, Inc., Cary, NC, BLS-106-350) and triethylamine in DMF.

EXAMPLE 86
Methoxy(polyethyleneoxy)$_n$-i-ethyl 3-Aminosulfonyl-4-phenoxy-5-(l-
pyrrolidinyl)thiobenzoate (S-Piretanide mPEG350 Thiosters)

In similar manner to Example 8, thiopiretanide can be reacted with MeO-PEG350-
Cl (Biolink Life Sciences, Inc., Cary, NC, BLS-106-350) and triethylamine in DMF to yield
methoxy(polyethyleneoxy)$_n$-i-ethyl 3-aminosulfonyl-4-phenoxy-5-(l-
pyrrolidinyl)thiobenzoate where n is in the 7-8 range.
EXAMPLE 87

Methoxy(polyethyleneoxy)\_n - i-ethyl 3-Aminosulfonyl-4-phenoxy-5-(1-pyrrolidinyl)benzoate (Piretanide mPEG1000 Esters)

In similar manner to Example 8, piretanide can be reacted with MeO-PEG1000-OTs (Biolink Life Sciences, Inc., Cary, NC, BLS-107-1000) and triethylamine in DMF to yield methoxy(polyethyleneoxy)\_n - i-ethyl 3-aminosulfonyl-4-phenoxy-5-(1-pyrrolidinyl)benzoate where n is in the 19-24 range. In similar manner S-piretanide mPEG1000 thiosters can be formed with S-thiopiretanide, MeO-PEG1000-OTs (Biolink Life Sciences, Inc., Cary, NC, BLS-107-1000) and triethylamine in DMF.

EXAMPLE 88

Methoxy(polyethyleneoxy)\_n - i-ethyl 3-Aminosulfonyl-4-phenoxy-5-(1-pyrrolidinyl)benzoate (Piretanide mPEG1000 Dithioesters)

In similar manner to Example 8, dithiopiretanide can be reacted with MeO-PEG1000-OTs (Biolink Life Sciences, Inc., Cary, NC, BLS-107-1000) and triethylamine in DMF to yield methoxy(polyethyleneoxy)\_n - i-ethyl 3-aminosulfonyl-4-phenoxy-5-(1-pyrrolidinyl)dithiobenzoate where n is in the 19-24 range. In similar manner piretanide mPEG1000 dithiosters can be formed with dithiopiretanide, MeO-PEG1000-OTs (Biolink Life Sciences, Inc., Cary, NC, BLS-107-1000) and triethylamine in DMF.

EXAMPLE 89

Methoxy(polyethyleneoxy)\_n - i-ethyl 5-Aminosulfonyl-4-chloro-2-(2-furanylmethyl)aminobenzoate (Furosemide mPEG350 Esters)

In similar manner to Example 8, furosemide can be reacted with MeO-PEG350-Cl (Biolink Life Sciences, Inc., Cary, NC, BLS-106-350) and triethylamine in DMF to yield methoxy(polyethyleneoxy)\_n - i-ethyl 5-aminosulfonyl-4-chloro-2-[(2-furanylmethyl)amino]benzoate where n is in the 7-8 range.

EXAMPLE 90

Methoxy(polyethyleneoxy)\_n - i-ethyl 5-Aminosulfonyl-4-chloro-2-[(2-furanylmethyl)amino]benzoate (S-Furosemide mPEG350 Thioesters)

In similar manner to Example 8, thiofurosemide can be reacted with MeO-PEG350-C1 (Biolink Life Sciences, Inc., Cary, NC, BLS-106-350) and triethylamine in DMF to yield methoxy(polyethyleneoxy)\_n - i-ethyl 5-aminosulfonyl-4-chloro-2-[(2-furanylmethyl)amino]thiobenzoate where n is in the 7-8 range.
EXAMPLE 91

Methoxy(polyethyleneoxy)n,i-ethyl 5-Aminosulfonyl-4-chloro-2-furanylmethyldiaminobenzoate (Furosemide mPEG1000 Esters)

In similar manner to Example 8, furosemide can be reacted with MeO-PEG1000-OTs (Biolink Life Sciences, Inc., Cary, NC, BLS-107-1000) and triethylamine in DMF to yield methoxy(polyethyleneoxy)n,i-ethyl 5-aminosulfonyl-4-chloro-2-furanylmethylamino]benzoate where n is in the 19-24 range.

EXAMPLE 92

Methoxy(polyethyleneoxy)^-ethyl 5-Aminosulfonyl-4-chloro-2-r(2-furanylmethyldiaminobenzoate (Furosemide mPEG1000 Dithioesters)

In similar manner to Example 8, dithiofurosemide can be reacted with MeO-PEG1000-OTs (Biolink Life Sciences, Inc., Cary, NC, BLS-107-1000) and triethylamine in DMF to yield methoxy(polyethyleneoxy)n-iethyl 5-aminosulfonyl-4-chloro-2-furanylmethylamino)dithiobenzoate where n is in the 19-24 range. In similar manner furosemide mPEG350 dithiosters can be formed with dithiofurosemide, MeO-PEG350-Cl (Biolink Life Sciences, Inc., Cary, NC, BLS-106-350) and triethylamine in DMF.

EXAMPLE 93

5-[l-Methoxy(polyethyleneoxy)n-i-ethyH-tetrazol-5-vI]-2-chloro-4-[2-thienylmethyl]aminobenzenesulfonamides (N-mPEG350-TetrazoM-Substituted Azosemides)

In similar manner to Example 8, azosemide can be reacted with MeO-PEG350-Cl (Biolink Life Sciences, Inc., Cary, NC, BLS-106-350) and triethylamine in DMF to yield 5-[l-[methoxy(polyethyleneoxy)n-i-ethyl]IH-tetrazol-5-yl]-2-chloro-4-[2-thienylmethyl]amino]benzenesulfonamides where n is in the 7-8 range.

EXAMPLE 94

5-[l-[Methoxy(polyethyleneoxy)n-i-ethyH-tetrazol-5-yl]2-chloro-4-f(2-thienylmethyl)amino]benzenesulfonamides (N-mPEG1000-TetrazoM-Substituted Azosemides)

In similar manner to Example 8, azosemide can be reacted with MeO-PEG1000-OTs (Biolink Life Sciences, Inc., Cary, NC, BLS-107-1000) and triethylamine in DMF to yield 5-[l-[methoxy (polyethyleneoxy)n-i-ethyl]-IH-tetrazol-5-yl]-2-chloro-4-[2-thienylmethyl]amino]benzenesulfonamides where n is in the 19-24 range.
EXAMPLE 95

\[ 3\text{''} \text{Isopropylcarbamylsulfonamido-4-(3'-methylphenyl)aminopyridinium} \]
\[ \text{Methoxy(polyethyleneoxy)n-i-ethochlorides (N-mPEG350-Pyridinium Torsemide Salts)} \]

In similar manner to Example 8, torsemide can be reacted with MeO-PEG350-Cl (Biolink Life Sciences, Inc., Gary, NC, BLS-106-350) and triethylamine in DMF to yield 3-isopropylcarbamylsulfonamido-4-(3'-methylphenyl)aminopyridinium methoxy(polyethyleneoxy)n-i-ethochlorides where \( n \) is in the 7-8 range.

EXAMPLE 96

\[ 3\text{-Isopropylcarbamylsulfonamido-4-(3'-methylphenyl)aminopyridinium} \]
\[ \text{Methoxy(polyethyleneoxy)n-i-ethochlorides (N-mPEG1000-Pyridinium Torsemide Salts)} \]

In similar manner to Example 8, torsemide can be reacted with MeO-PEG1000-OTs (Biolink Life Sciences, Inc., Cary, NC, BLS-107-1000) and triethylamine in DMF to yield 3-isopropylcarbamylsulfonamido-4-(3'-methylphenyl)aminopyridinium methoxy(polyethyleneoxy)n-i-ethochlorides where \( n \) is in the 19-24 range.

EXAMPLE 97

\[ 3\text{-Aminosulfonyl-5-butylamino-4-phenoxybenzaldehyde (Buinetanide Aldehyde)} \]

By the method of Muraki and Mukiyama \( \text{(Chem. Letters, 1975, 215)} \), bumetanide can be reacted with bis(4-methylpiperazinyl)aluminum hydride to yield 3-aminosulfonyl-5-butylamino-4-phenoxybenzaldehyde.

EXAMPLE 98

\[ 3\text{-Aminosulfonyl-4-phenoxy-5-(l-pyrrolidinyl)benzaldehyde (Piretanide Aldehyde)} \]

By the method of Muraki and Mukiyama \( \text{(Chem. Letters, 1975, 215)} \), piretanide can be reacted with bis(4-methylpiperazinyl)aluminum hydride to yield 3-aminosulfonyl-4-phenoxy-5-(1-pyrrolidinyl)benzaldehyde.

EXAMPLE 99

\[ 5\text{-Aminosulfonyl-4-chloro-2-[(2-furanylmethyl)amino]benzaldehyde (Furosemide Aldehyde)} \]

By the method of Muraki and Mukiyama \( \text{(Chem. Letters, 1974, 1447 and Chem. Letters, 1975, 215)} \), furosemide can be reacted with bis(4-methylpiperazinyl)aluminum hydride to yield 5-aminosulfonyl-4-chloro-2-[(2-furanylmethyl)amino]benzaldehyde.
EXAMPLE 100

The Effects of Furosemide on Epileptiform Discharges in Hippocampal Slices

During these studies, spontaneous epileptiform activity was elicited by a variety of treatments. Sprague-Dawley rats (males and females; 25-35 days old) were decapitated, the top of the skull was rapidly removed, and the brain chilled with ice-cold oxygenated slicing medium. The slicing medium was a sucrose-based artificial cerebrospinal fluid (sACSF) consisting of 220 mM sucrose, 3 nM KCl, 1.25 mM NaH₂PO₄, 2 mM MgSO₄, 26 mM NaHCO₃, 2 mM CaCl₂, and 10 mM dextrose (295-305 mOsm). A hemisphere of brain containing hippocampus was blocked and glued (cyanoacrylic adhesive) to the stage of a Vibroslicer (Frederick Haer, Brunsick, ME). Horizontal or transverse slices 400 µm thick were cut in 4°C, oxygenated (95% O₂; 5% CO₂) slicing medium. The slices were immediately transferred to a holding chamber where they remained submerged in oxygenated bathing medium (ACSF) consisting of 124 mM NaCl, 3 mM KCl, 1.25 mM NaH₂PO₄, 2 mM MgSO₄, 26 mM NaHCO₃, 2 mM CaCl₂, and 10 mM dextrose (295-305 mOsm). The slices were held at room temperature for at least 45 minutes before being transferred to a submersion-style recording chamber (all other experiments). In the recording chamber, the slices were perfused with oxygenated recording medium at 34-35°C. All animal procedures were conducted in accordance with NIH and University of Washington animal care guidelines.

In most slice experiments, simultaneous extracellular field electrode recordings were obtained from CA1 and CA3 areas. A bipolar tungsten stimulating electrode was placed on the Schaffer collaterals to evoke synaptically-driven field responses in CA1. Stimuli consisted of 100-300 µsec duration pulses at an intensity of four times the population-spike threshold. After discharges were evoked by a 2 second train of such stimuli delivered at 60 Hz. Spontaneous interictal-like bursts were observed in slices treated by the following modifications or additions to the bathing medium: 10 mM potassium (6 slices; 4 animals; average - 81 bursts/min.); 200-300 µM 4-aminopyridine (4 slices; 2 animals; average —33 burst/min.); 50-100 µM bicuculline (4 slices; 3 animals; average - 14 bursts/min); M Mg²⁺ (1 hour of perfusion - 3 slices; 2 animals; average - 20 bursts/min. or 3 hours of perfusion - 2 slices; 2 animals); zero calcium/6 mM KCl and 2 mM EGTA (4 slices; 3 animals). In all treatments, furosemide was added to the recording medium once a consistent level of bursting was established.
In the first of these procedures, episodes of after discharges were evoked by electrical stimulation of the Schaffer collaterals (Stasheff et al, *Brain Res.* 344:296, 1985) and the extracellular field response was monitored in the CA1 pyramidal cell region (13 slices; 8 animals). The concentration of Mg$^{2+}$ in the bathing medium was reduced to 0.9 mM and after discharges were evoked by stimulation at 60 Hz for 2 seconds at an intensity 4 times the population spike threshold (population spike threshold intensity varied between 20-150 µA at 100-300 µsec pulse duration). The tissue was allowed to recover for 10 minutes between stimulation trials. In each experiment, the initial response of CA1 to synaptic input was first tested by recording the field potential evoked by a single stimulus pulse. In the control condition, Schaffer collateral stimulation evoked a single population spike (Fig. IA, inset). Tetanic stimulation evoked approximately 30 seconds after discharge (Fig. IA, left) associated with a large change in intrinsic signal (Fig. IA, right).

For imaging of intrinsic optical signals, the tissue was placed in a perfusion chamber located on the stage of an upright microscope and illuminated with a beam of white light (tungsten filament light and lens system; Dedo Inc.) directed through the microscope condenser. The light was controlled and regulated (power supply - Lambda Inc.) to minimize fluctuations and filtered (695 nm longpass) so that the slice was transilluminated with long wavelengths (red). Field of view and magnification were determined by the choice of microscope objectives (4X for monitoring the entire slice).

Image-frames were acquired with a charge-coupled device (CCD) camera (Dage MTI Inc.) at 30 HZ and were digitized at 8 bits with a spatial resolution of 512 x 480 pixels using an Imaging Technology Inc. Series 151 imaging system; gains and offsets of the camera-control box and the A/D board were adjusted to optimize the sensitivity of the system. Imaging hardware was controlled by a 486-PC compatible computer. To increase signal/noise, an averaged-image was composed from 16 individual image-frames, integrated over 0.5 sec and averaged together. An experimental series typically involved the continuous acquisition of a series of averaged-images over a several minute time period; at least 10 of these averaged-images were acquired as control-images prior to stimulation. Pseudocolored images were calculated by subtracting the first control-image from subsequently acquired images and assigning a color lookup table to the pixel values. For these images, usually a linear low-pass filter was used to remove high frequency noise and a linear-histogram stretch was used to map the pixel values over the dynamic.
range of the system. All operations on these images were linear so that quantitative
information was preserved. Noise was defined as the maximum standard deviation of
fluctuations of AR/R of the sequence of control images within a given acquisition series,
where AR/R represented the magnitude of the change in light-transmission through the
tissue. Delta R/R was calculated by taking all the difference-images and dividing by the
first control image: (subsequent image - first-control-image)/first-control-image. The
noise was always <0.01 for each of the chosen image sequences. The absolute change in
light transmission through the tissue was estimated during some experiments by acquiring
images after placing neutral density filters between the camera and the light source. On
average, the camera electronics and imaging system electronics amplified the signal 10-
fold prior to digitization so that the peak absolute changes in light transmission through
the tissue were usually between 1% and 2%.

The gray-scale photo shown in Fig. 1D is a video image of a typical hippocampal
slice in the recording chamber. The fine gold-wire mesh that was used to hold the tissue
in place can be seen as dark lines running diagonally across the slice. A stimulating
electrode can be seen in the upper right on the stratum radiatum of CA1. The recording
electrode (too thin to be seen in the photo) was inserted at the point indicated by the white
arrow. Fig. IA illustrates that two seconds of stimulation at 60 Hz elicited after discharge
activity and shows a typical after discharge episode recorded by the extracellular
electrode. The inset of Fig. IA shows the CA1 field response to a single 200 sec test
pulse (artifact at arrow) delivered to the Schaffer collaterals. Fig. IAI shows a map of
the peak change in optical transmission through the tissue evoked by Schaffer collateral
stimulation. The region of maximum optical change corresponds to the apical and basal
dendritic regions of CA1 on either side of the stimulating electrode. Fig. IB illustrates
sample traces showing responses to stimulation after 20 minutes of perfusion with
medium containing 2.5 mM furosemide. Both the electrical after discharge activity
(shown in Fig. IB) and the stimulation-evoked optical changes (shown in Fig. IBI) were
blocked. However, there was a hyper-excitable field response (multiple population
spikes) to the test pulse (inset). Figs 1C and 1CI illustrate that restoration of initial
response patterns was seen after 45 minutes of perfusion with normal bathing medium.

The opposing effects of furosemide-blockade of the stimulation-evoked after
discharges and a concomitant increase of the synaptic response to a test-pulse illustrate
the two key results: (1) furosemide blocked epileptiform activity, and (2) synchronization
(as reflected by spontaneous epileptiform activity) and excitability (as reflected by the
response to a single synaptic input) were dissociated. Experiments in which the dose
dependency of furosemide was examined determined that a minimum concentration of
1.25 mM was required to block both the after discharges and optical changes.

EXAMPLE 101

The effects of furosemide on epileptiform discharges in hippocampal slices
perfused with high-K+ (10 mM) bathing medium

Rat hippocampal slices, prepared as described above, were perfused with a high-
K+ solution until extended periods of spontaneous interictal-like bursting were recorded
simultaneously in CA3 (top traces) and CA1 (lower traces) pyramidal cell regions (Figs.
2A and 2B). After 15 minutes of perfusion with furosemide-containing medium (2.5 mM
furosemide), the burst discharges increased in magnitude (Figs. 2C and 2D). However,
after 45 minutes of furosemide perfusion, the bursts were blocked in a reversible manner
(Figs 2E, 2F, 2G and 2H). During this entire sequence of furosemide perfusion, the
synaptic response to a single test pulse delivered to the Schaffer colaterals was either
unchanged or enhanced (data not shown). It is possible that the initial increase in
discharge amplitude reflected a furosemide-induced decrease in inhibition (Misgeld et al.,
Science 232:1413, 1986; Thompson et al., J. Neurophysiol 60:105, 1988; Thompson and
previously been reported (Pearce, Neuron 10:189, 1993) that furosemide blocks a
component of the inhibitory currents in hippocampal slices with a latency (<15 min.)
similar to the time to onset of the increased excitability observed here. The longer latency
required for the furosemide-block of the spontaneous bursting might correspond to
additional time required for a sufficient block of the furosemide-sensitive cellular volume
regulation mechanisms under high-K+ conditions.

After testing the effects of furosemide on slices perfused with high-K+, similar
studies were performed with a variety of other commonly studied in vitro models of
epileptiform discharge (Galvan et al., Brain Res. 241:75, 1982; Schwartzkroin and Prince,
Brain Res.183:61, 1980; Anderson et al., Brain Res. 398:215, 1986; and Zhang et al.,
Epilepsy Res. 20:105, 1995). After prolonged exposure (2-3 hours) to magnesium-free
medium (0-Mg⁻), slices have been shown to develop epileptiform discharges that are
resistant to common clinically used anticonvulsant drugs (Zhang et al., Epilepsy Res.
20:105, 1995). Recordings from entorhinal cortex (Fig. 21) and subiculum (not shown)
showed that after 3 hours of perfusion with 0-Mg⁺⁺ medium, slices developed bursting
patterns that appeared similar to these previously described "anticonvulsant resistant" bursts. One hour after the addition of furosemide to the bathing medium, these bursts were blocked (Fig. 2J). Furosemide also blocked spontaneous burst discharges observed with the following additions/modifications to the bathing medium: (1) addition of 200-300 µM 4-aminopyridine (4-AP; a potassium channel blocker) (Figs. 2K and 2L); (2) addition of the GABA antagonist, bicuculline, at 50-100 µM (Figs. 2M ad 2N); (3) removal of magnesium (0-Mg++) - 1 hours perfusion (Figs. 20 and 2P); and (4) removal of calcium plus extracellular chelation (0-Ca++) (Figs. 2Q and 2R). With each of these manipulations, spontaneous interictal-like patterns were simultaneously recorded from CA1 and CA3 subfields (Figs. 2K, 2L, 2M and 2N show only the CA3 trace and Figs. 20, 2P, 2Q, and 2R show only the CA1 trace). In the 0-Ca++ experiments, 5 mM furosemide blocked the bursting with a latency of 15-20 minutes. For all other protocols, bursting was blocked by 2.5 mM furosemide with a latency of 20-60 minutes. Furosemide reversibly blocked the spontaneous bursting activity in both CA1 and CA3 in all experiments (Figs 2L, 2N, 2P and 2R).

EXAMPLE 102

**The effects of furosemide on epileptiform activity induced by i.v. injection of kainic acid in anesthetized rats**

This example illustrates an *in vivo* model in which epileptiform activity was induced by i.v. injection of kainic acid (KA) into anesthetized rats (Lothman et al., *Neurology* 31:806, 1981). The results are illustrated in Figs. 3A - 3H. Sprague-Dawley rats (4 animals; weights 250-270 g) were anesthetized with urethane (1.25 g/kg i.p.) and anesthesia maintained by additional urethane injections (0.25 g/kg i.p.) as needed. Body temperature was monitored using a rectal temperature probe and maintained at 35-37°C with a heating pad; heart rate (EKG) was continuously monitored. The jugular vein was cannulated on one side for intravenous drug administration. Rats were placed in a Kopf stereotaxic device (with the top of the skull level), and a bipolar stainless-steel microelectrode insulated to 0.5 mm of the tip was inserted to a depth of 0.5-1.2 mm from the cortical surface to record electroencephalographic (EEG) activity in the fronto-parietal cortex. In some experiments, a 2M NaCl-containing pipette was lowered to a depth of 2.5-3.0 mm to record hippocampal EEG. Data were stored on VHS videotape and analyzed off-line.
Following the surgical preparation and electrode placement, animals were allowed to recover for 30 minutes before the experiments were initiated with an injection of kainic acid (10-12 mg/kg i.v.). Intense seizure activity, an increased heart rate, and rapid movements of the vibrissae were induced with a latency of about 30 minutes. Once stable electrical seizure was evident, furosemide was delivered in 20 mg/kg boluses every 30 minutes to a total of 3 injections. Experiments were terminated with the intravenous administration of urethane. Animal care was in accordance with NIH guidelines and approved by the University of Washington Animal Care Committee.

Figs. 3A-3H show furosemide blockade of kainic acid-evoked electrical "status epilepticus" in urethane-anesthetized rats. EKG recordings are shown as the top traces and EEG recordings are shown as the bottom traces. In this model, intense electrical discharge (electrical "status epilepticus") was recorded from the cortex (or from depth hippocampal electrodes) 30-60 minutes after KA injection (10-12 mg/kg) (Figs. 3C and 3D). Control experiments (and previous reports, Lothman et al., Neurology, 31:806, 1981) showed that this status-like activity was maintained for well over 3 hours. Subsequent intravenous injections of furosemide (cumulative dose: 40-60 mg/kg) blocked seizure activity with a latency of 30-45 minutes, often producing a relatively flat EEG (Figs. 3E, 3F, 3G and 3H). Even 90 minutes after the furosemide injection, cortical activity remained near normal baseline levels (i.e., that observed prior to the KA and furosemide injections). Studies on the pharmacokinetics of furosemide in the rat indicate that the dosages used in this example were well below toxic levels (Hammarlund and Paalzow, Biopharmaceutics Drug Disposition, 3:345, 1982).

**Experimental methods for Examples 103 - 106**

Hippocampal slices were prepared from Sprague-Dawley adult rats as described previously. Transverse hippocampal slices 100 µm thick were cut with a vibrating cutter. Slices typically contained the entire hippocampus and subiculum. After cutting, slices were stored in an oxygenated holding chamber at room temperature for at least one hour before recording. AU recordings were acquired in an interface type chamber with oxygenated (95% O₂, 5%CO₂) artificial cerebral spinal fluid (ACSF) at 34°-35°C. Normal ACSF contained (in mmol/l): 124 NaCl, 3 KCl, 1.25 NaH₂PO₄, 1.2 MgSO₄, 26 NaHCO₃, 2 CaCl₂, and 10 dextrose.
Sharp-electrodes for intracellular recordings from CA1 and CA3 pyramidal cells were filled with 4 M potassium acetate. Field recordings from the CA1 and CA3 cell body layers were acquired with low-resistance glass electrodes filled with 2 M NaCl. For stimulation of the Schaffer collateral or hilar pathways, a small monopolar tungsten electrode was placed on the surface of the slice. Spontaneous and stimulation-evoked activities from field and intracellular recordings were digitized (Neurocorder, Neurodata Instruments, New York, NY) and stored on videotape. AxoScope software (Axon Instruments) on a personal computer was used for off-line analysis of data.

In some experiments, normal or low-chloride medium was used containing bicuculline (20 µM), 4-amino pyridine (4-AP) (100 µM), or high-K+ (7.5 or 12 mM). In all experiments, low-chloride solutions (7, and 21 mM [Cl⁻]) were prepared by equimolar replacement of NaCl with Na⁺-gluconate (Sigma). All solutions were prepared so that they had a pH of approximately 7.4 and an osmolality of 290-300 mOsm at 35°C and at equilibrium from carboxygenation with 95%O₂ / 5%CO₂.

After placement in the interface chamber, slices were superfused at approximately 1 ml/min. At this flow-rate, it took 8-10 minutes for changes in the perfusion media to be completed. All of the times reported here have taken this delay into account and have an error of approximately ± 2 minutes.

**EXAMPLE 103**

**Timing of cessation of spontaneous epileptiform bursting in areas in CA1 and CA3**

The relative contributions of the factors that modulate synchronized activity vary between areas CA1 and CA3. These factors include differences in the local circuitry and region-specific differences in cell packing and volume fraction of the extracellular spaces. If the anti-epileptic effects of anion or chloride-cotransport antagonism are due to a desynchronization in the timing of neuronal discharge, chloride-cotransport blockade might be expected to differentially affect areas CA1 and CA3. To test this, a series of experiments was performed to characterize differences in the timing of the blockage of spontaneous epileptiform activity in areas CA1 and CA3.

Field activity was recorded simultaneously in areas CA1 and CA3 (approximately midway between the most proximal and distal extent the CA3 region), and spontaneous bursting was induced by treatment with high-[K⁺]O (12 µM; n = 12), bicuculline (20 mM; n = 12), or 4-AP (100 µM; n = 5). Single electrical stimuli were delivered to the Schaffer
collaterals, midway between areas CA1 and CA3, every 30 seconds so that the field responses in areas CA1 and CA3 could be monitored throughout the duration of each experiment. In all experiments, at least 20 minutes of continuous spontaneous epileptiform bursting was observed prior to switching to low [CF]O (21 mM) or furosemide-containing (2.5 mM) medium.

In all cases, after 30-40 minutes exposure to furosemide or low-chloride medium, spontaneous bursting ceased in area CA1 before the bursting ceased in area CA3. The temporal sequence of events typically observed included an initial increase in burst frequency and amplitude of the spontaneous field events, then a reduction in the amplitude of the burst discharges which was more rapid in CA1 than in CA3. After CA1 became silent, CA3 continued to discharge for 5-10 minutes, until it too no longer exhibited spontaneous epileptiform events.

This temporal pattern of burst cessation was observed with all epileptiform-inducing treatments tested, regardless of whether the agent used for blockade of spontaneous bursting was furosemide or low-\([\text{Cl}]\)O medium. Throughout all stages of these experiments, stimulation of the Schaffer collaterals evoked hyperexcited field responses in both the CA1 and CA3 cell body layers. Immediately after spontaneous bursting was blocked in both areas CA1 and CA3, hyperexcited population spikes could still be evoked.

We considered the possibility that the observed cessation of bursting in CA1 prior to CA3 was an artifact of the organization of synaptic contacts between these areas relative to our choice of recording sites. It is known that the projections of the various subregions of CA3 terminate in an organized fashion in CA1; CA3 cells closer to the dentate gyrus (proximal CA3) tend to project most heavily to the distal portions of CA1 (near the subicular border), whereas CA3 projections arising from cells located more distally in CA3 terminate more heavily in portions of CA1 located closer to the CA2 border. If the cessation of bursting occurs in the different subregions of CA3 at different times, the results of the above set of experiments might arise not as a difference between CA1 and CA3, but rather as a function of variability in bursting activity across CA3 subregions. We tested this possibility in three experiments. Immediately after the spontaneous bursting ceased in CA1, we surveyed the CA3 field with a recording electrode. Recordings from several different CA3 locations (from the most proximal to the most distal portions of CA3), showed that all subregions of area CA3 were spontaneously bursting during the time that CA1 was silent.
The observation that CA3 continued to discharge spontaneously after CA1 became silent was unexpected since population discharges in CA3 are generally thought to evoke discharges in CA1 through excitatory synaptic transmission. As previously described, single-pulse stimuli delivered to the Schaffer collaterals still evoked multiple population spikes in CA1 even after the blockade of spontaneous bursting; thus, hyperexcited excitatory synaptic transmissions in CA3-to-CA1 synapse was intact. Given this maintained efficacy of synaptic transmission, and the continued spontaneous field discharges in CA3, we postulated that the loss of spontaneous bursting in CA1 was due to a decrease in synchronization of incoming excitatory drive. Further, since spontaneous epileptiform discharge in CA3 also eventually ceased, perhaps this desynchronization process occurred at different times in the two hippocampal subfields.

EXAMPLE 104
Effect of chloride-cotransport antagonism on the synchronization of CA1 and CA3 field population discharges

The observation from Example 103 suggested a temporal relationship between the exposure time to low-[CF]O or furosemide-containing medium and the characteristics of the spontaneous burst activity. Further, this relationship was different between areas CA1 and CA3. In order to better characterize the temporal relationships, we compared the occurrences of CA1 action potentials and the population spike events in the field responses of CA1 and CA3 subfields during spontaneous and stimulation-evoked burst discharge.

Intracellular recordings were obtained from CA1 pyramidal cells, with the intracellular electrode placed close (<100 µM) to the CA1 field electrode. The slice was stimulated every 20 seconds with single stimuli delivered to the Schaffer collaterals.

After continuous spontaneous bursting was established for at least 20 minutes, the bathing medium was switched to bicuculline-containing low-[Cl]O (21 mM) medium. After approximately 20 minutes, the burst frequency and amplitude was at its greatest. Simultaneous field and intracellular recordings during this time showed that the CA1 field and intracellular recordings were closely synchronized with the CA3 field discharges. During each spontaneous discharge, the CA3 field response preceded the CA1 discharge by several milliseconds. During stimulation-evoked events, action potential discharges of the CA1 pyramidal cell were closely synchronized to both CA3 and CA1 field discharges.
With continued exposure to low-[Cl\textsuperscript{−}]O medium, the latency between the spontaneous discharges of areas CA1 and CA3 increased, with a maximum latency of 30-40 milliseconds occurring after 30-40 minutes exposure to the bicuculline-containing low-chloride medium. During this time, the amplitude of both the CA1 and CA3 spontaneous field discharges decreased. Stimulation-evoked discharges during this time closely mimicked the spontaneously occurring discharges in morphology and relative latency. However, the initial stimulus-evoked depolarization of the neuron (presumably, the monosynaptic EPSP) began without any significant increase in latency. The time interval during which these data were acquired corresponds to the time immediately prior to the cessation of spontaneous bursting in CA1.

After 40-50 minutes perfusion with low-[Cl\textsuperscript{−}]O medium, the spontaneous bursts were nearly abolished in CA1 but were unaffected in CA3. Schaffer collateral stimulation during this time showed that monosynthetically-triggered responses of CA1 pyramidal cells occurred without any significant increase in latency, but that stimulation-evoked field responses were almost abolished. The time interval during which these data were acquired corresponds to the moments immediately prior to the cessation of spontaneous bursting in CA3.

After prolonged exposure to low-[CF\textsuperscript{−}]O medium, large increases (>30 milliseconds) developed in the latency between Schaffer collateral stimulation and the consequent CA3 field discharge. Eventually, no field responses could be evoked by Schaffer collateral stimulation in either areas CA1 and CA3. However, action potential discharge from CA1 pyramidal cells in response to Schaffer collateral stimulation could be evoked with little change in response latency. Indeed, for the entire duration of the experiments (greater than two hours), action potential discharges form CA1 pyramidal cells could be evoked at short latency by Schaffer collateral stimulation. Further, although stimulation-evoked hyperexcited discharges of CA3 were eventually blocked after prolonged exposure to low-[Cl\textsuperscript{−}]O medium, the antidromic response in CA3 appeared to be preserved.

**EXAMPLE 105**

**Effects of chloride-cotransport antagonism on the synchronization of burst discharges in CA1 pyramidal cells**

The foregoing data suggest the disappearance of the field responses may be due to a desynchronization of the occurrence of action potentials among neurons. That is,
although synaptically-driven excitation of CA1 pyramidal cells was not preserved, action potential synchrony among the CA1 neuronal population was not sufficient to summate into a measurable DC field response. In order to test this, paired intracellular recordings of CA1 pyramidal cells were acquired simultaneously with CA1 field responses. In these experiments, both the intracellular electrodes and the field recording electrodes were placed within 200 µm of one another.

During the period of maximum spontaneous activity induced by bicuculline-containing low-[Cl\(^-\)]O medium, recordings showed that action potentials between pairs of CA1 neurons and the CA1 field discharges were tightly synchronized both during spontaneous and stimulation-evoked discharges. After continued exposure to low-[Cl\(^-\)]O medium, when the amplitude of the CA1 field discharge began to broaden and diminish, both spontaneous and stimulation-evoked discharges showed a desynchronization in the timing of the occurrences of action potentials between pairs of CA1 neurons, and between the action potentials and the field responses. This desynchronization was coincident with the suppression of CA1 field amplitude. By the time that spontaneous bursting in CA1 ceased, a significant increase in latency had developed between Schaffer collateral stimulation and CA1 field discharge. At this time, paired intracellular recordings showed a dramatic desynchronization in the timing of action potential discharge between pairs of neurons and between the occurrence of action potentials and the field discharges evoked by Schaffer collateral stimulation.

It is possible that the observed desynchronization of CA1 action potential discharge is due to the randomization of mechanisms necessary for synaptically-driven action potential generation, such as a disruption in the timing of synaptic release or random conduction failures at neuronal processes. If this were the case, then one would expect that the occurrence of action potentials between a given pair of neurons would vary randomly with respect to one another, from stimulation to stimulation. We tested this by comparing the patterns of action potential discharge of pairs of neurons between multiple consecutive stimuli of the Schaffer collaterals. During each stimulation event, the action potentials occurred at nearly identical times with respect to one another, and showed an almost identical burst morphology from stimulation to stimulation. We also checked to see whether the occurrence of action potentials between a given pair of neurons during spontaneous field discharges was fixed in time. The patterns of action potential discharges from a given pair of CA1 neurons was compared between
consecutive spontaneous field bursts during the time when the occurrence of action potentials was clearly desynchronized. Just as in the case of stimulation-evoked action potential discharge described above, the action potentials generated during a spontaneous population discharge occurred at nearly identical times with respect to one another, and showed a nearly identical burst morphology from one spontaneous discharge to the next.

EXAMPLE 106

Effects of low-chloride treatment on spontaneous synaptic activity

It is possible that the anti-epileptic effects associated with chloride-cotransport antagonism are mediated by some action on transmitter release. Blockade of chloride-cotransport could alter the amount or timing of transmitter released from terminals, thus affecting neuronal synchronization. To test whether low-[Cl]\textsuperscript{-}O exposure affected mechanisms associated with transmitter release, intracellular CA1 responses were recorded simultaneously with CA1 and CA3 field responses during a treatment which dramatically increases spontaneous synaptic release of transmitter from presynaptic terminals.

Increased spontaneous release of transmitter was induced by treatment with 4-AP (100 μM). After 40 minutes exposure to 4-AP-containing medium, spontaneous synchronized burst discharges were recorded in area CA1 and CA3. Switching to 4-AP-containing low-[Cl]\textsuperscript{-}O medium led initially, as was shown previously, to enhanced spontaneous bursting. High-grain intracellular recordings showed that high-amplitude spontaneous synaptic activity was elicited by 4-AP treatment. Further exposure to low-chloride medium blocked spontaneous burst discharge in CA1, although CA3 continued to discharge spontaneously. At this time, CA1 intracellular recordings showed that spontaneous synaptic noise was further increased, and remained so for prolonged exposure times to 4-AP-containing low-chloride medium. These data suggest that mechanisms responsible for synaptic release from terminals are not adversely affected by low-chloride exposure in a manner that could explain the blockade of 4-AP-induced spontaneous bursting in CA1. These results also eliminate the possibility that the effects of low-[Cl]\textsuperscript{-}O exposure are due to alterations in CA1 dendritic properties which would compromise their efficiency in conducting PSPs to the soma.
Experimental Methods for Examples 107 to 111

In all of the following experiments, [CT]O was reduced by equimolar replacement of NaCl with Na\(^+\)-gluconate. Gluconate was used rather than other anion replacements for several reasons. First, patch-clamp studies have demonstrated that gluconate appears to be virtually impermeant to chloride channels, whereas other anions (including sulfate, isethionate, and acetate) are permeable to varying degrees. Second, transport of extracellular potassium through glial NKCC\(_1\) cotransport is blocked when extracellular chloride is replaced by gluconate but is not completely blocked when replaced by isethionate. Since this furosemide-sensitive cotransporter plays a significant role in cell swelling and volume changes of the extracellular space (ECS), we wished to use the appropriate anion replacement so that the effects of our treatment would be comparable to previous furosemide experiments (Hochman et al. Science, 270:99-102, 1995; US Patent No. 5,902,732). Third, formate, acetate, and propionate generate weak acids when employed as Cl\(^-\) substitutes and lead to a prompt fall in intracellular pH; gluconate remains extracellular and has not been reported to induce intracellular pH shifts. Fourth, for purposes of comparison we wished to use the same anion replacement that had been used in previous studies examining the effects of low-[CF]O on activity-evoked changes of the ECS.

There is some suggestion that certain anion-replacements might chelate calcium. Although subsequent work has failed to demonstrate any significant ability of anion-substitutes to chelate calcium, there is still some concern in the literature regarding this issue. Calcium chelation did not appear to be an issue in the following experiments, since resting membrane potentials remained normal and synaptic responses (indeed, hyperexcitable synaptic responses) could be elicited even after several hours of exposure to medium in which [CF]O had been reduced by gluconate substitution. Further, we confirmed that calcium concentration in our low-[Cl\(^-\)]O -medium was identical to that in our control-medium by measurements made with Ca\(^{2+}\) - selective microelectrodes.

Sprague-Dawley adult rats were prepared as previously described. Briefly, transverse hippocampal slices, 400 \(\mu\)m thick, were cut using a vibrating cutter. Slices typically contained the entire hippocampus and subiculum. After cutting, slices were stored in an oxygenated holding chamber for at least one hour prior to recording. All recordings were acquired in an interface type chamber with oxygenated (95% 02/5% \(CO\(_2\)\)) artificial cerebral spinal fluid (ACSF) at 34°-35°C. Normal ACSF contained (in
mmol/1): 124 NaCl, 3 KCl, 1.25 NaH$_2$PO$_4$, 1.2 MgSO$_4$, 26 NaHCO$_3$, 2 CaCl$_2$, and 10
dextrose. In some experiments, normal or low-chloride medium was used containing
bicuculline (20 µM), 4-AP (100 µM), or high-K$^+$ (12 mM). Low-chloride solutions (7,
16, and 21 mM [CF]O) were prepared by equimolar replacement of NaCl with Na$^+$
gluconate (Sigma Chemical Co., St. Louis, MO). All solutions were prepared so that they
had a pH of approximately 7.4 and an osmolarity of 290-300 mOsm at 35°C and at
equilibrium from carboxygenation with 95% O$_2$ / 5% CO$_2$.

Sharp-electrodes filled with 4 M potassium acetate were used for intracellular
recordings from CA1 pyramidal cells. Field recordings from the CA1 or CA3 cell body
layers were acquired with low-resistance glass electrodes filled with NaCl (2 M). For
stimulation of the Schaffer collateral pathway, a small monopolar electrode was placed on
the surface of the slice midway between areas CA1 and CA3. Spontaneous and
stimulation-evoked activities from field and intracellular recordings were digitized
(Neurocorder, Neurodata Instruments, New York, NY), and stored on video tape.
AxoScope software (Axon Instruments Inc.) on a PC-computer was used for off-line
analyses of data.

Ion-selective microelectrodes were fabricated according to standard methods well
known in the art. Double-barreled pipettes were pulled and broken to a tip diameter of
approximately 3.0 µm. The reference barrel was filled with ACSF and the other barrel
was sylanized and the tip back-filled with a resin selective for K$^+$ (Corning 4773 17). The
remainder of the sylanized barrel was filled with KCl (140 mM). Each barrel was led, via
Ag/AgCl wires, to a high impedance dual-differential amplifier (WPI FD223). Each ion-
selective microelectrode was calibrated by the use of solutions of known ionic
composition and was considered suitable if it was characterized by a near-Nernstian slope
response and if it remained stable throughout the duration of the experiment.

After placement in the interface chamber, slices were superfused at approximately
1 ml/minute. At this flow-rate, it took approximately 8-10 minutes for changes in
perfusion media to be completed. All of the times reported here have taken this time-
delay into account and have an error of approximately ± 2 minutes.
EXAMPLE 107
Effects Of low-[Cl-IO on CA1 field recordings

Other studies have shown that prolonged exposure of cortical and hippocampal slices to low-[Cl']O does not affect basic intrinsic and synaptic properties such as input resistance, resting membrane potential, depolarization-induced action-potential generation, or excitatory synaptic transmission. A previous study has also partly characterized the epileptogenic properties of low-[CT]O exposure to the CA1 area of hippocampus. The following studies were performed to observe the times of onset and possible cessation of low-[Cr]O-induced hyperexcitability and hypersynchronization. Slices (n = 6) were initially perfused with normal medium until stable intracellular and field recordings were established in a CA1 pyramidal cell and the CA1 cell body layer, respectively. In two experiments, the same cell was held throughout the entire length of the experiment (greater than 2 hours). In the remaining experiments (n = 4), the initial intracellular recording was lost during the sequence of medium changes and additional recordings were acquired from different cells. Patterns of neuronal activity in these experiments were identical to those seen when a single cell was observed.

The field and intracellular electrodes were always placed in close proximity to one another (< 200 µm). In each case, after approximately 15-20 minutes exposure to the low-[Cr]O-medium (7 mM), spontaneous bursting developed, first at the cellular level, and then in the field. This spontaneous field activity, representing synchronized burst discharge in a large population of neurons, lasted from 5 - 10 minutes, after which time the field recording became silent. When the field first became silent, the cell continued to discharge spontaneously. This result suggests that population activity has been "desynchronized" while the ability of individual cells to discharge has not been impaired. After approximately 30 minutes exposure to low-[Cr]O-medium, intracellular recording showed that cells continued to discharge spontaneously even though the field remained silent. The response of the cell to intracellular current injection at two time points demonstrated that the cell's ability to generate action potentials had not been impaired by low-[Cl']O exposure. Further, electrical stimulation in CA1 stratum radiatum elicited burst discharges, indicating that a hyperexcitable state was maintained in the tissue.
**EXAMPLE 108**

**Effects of low-[Cl]-O on high-[K+]-O-induced epileptiform activity in CA1**

The previous set of experiments showed that tissue exposure to low-[Cl]-O medium induced a brief period of spontaneous field potential bursting which ceased within 10 minutes. If a reduction of [Cl]-O is indeed eventually capable of blocking spontaneous epileptiform (i.e. synchronized) bursting, then these results suggest that antiepileptic effects would likely be observable only after this initial period of bursting activity has ceased. We therefore examined the temporal effects of low-[Cl]-O-treatment on high-[K+]O-induced bursting activity. Slices (n = 12) were exposed to medium in which [K+]O had been increased to 12 mM, and field potentials were recorded with a field electrode in the CA1 cell body layer. Spontaneous field potential bursting was observed for at least 20 minutes, and then the slices were exposed to medium in which [K+]O was maintained at 12 mM, but [Cl]-O was reduced to 21 mM. Within 15-20 minutes after the tissue was exposed to the low-[Cl]-O/high-[K+]O-medium, the burst amplitude increased and each field event had a longer duration. After a brief period of this facilitated field activity (lasting 5-10 minutes), the bursting stopped. To test whether this blockade was reversible, after at least 10 minutes of field potential silence, we switched back to high-[K+]O-medium with normal [Cl]-O. The bursting returned within 20-40 minutes. Throughout each experiment, the CA1 field response to Schaffer collateral stimulation was monitored. The largest field responses were recorded just before the cessation of spontaneous bursting, during the period when the spontaneous bursts had the largest amplitude. Even after the blockade of spontaneous bursting, however, multiple population spikes were elicited by Schaffer collateral stimulation, indicating that synaptic transmission was intact, and that the tissue remained hyperexcitable.

In four slices, intracellular recordings from CA1 pyramidal cells were acquired along with the CA1 field recording. During the period of high-[K+]O-induced spontaneous bursting, hyperpolarizing current was injected into the cell so that postsynaptic potentials (PSPs) could be better observed. After low-[Cl]-O-blockade of spontaneous bursting, spontaneously occurring action potentials and PSPs were still observed. These observations further support the view that synaptic activity, per se, was not blocked by the low-[Cl]-O treatment.
EXAMPLE 109

Low-[CF]O - blockade of epileptiform activity induced by 4-AP, high-[K+]O, and bicuculline in CA1 and CA3

We next tested whether low-[CF]O treatment could block epileptiform activity in areas CA1 and CA3, which was elicited by different pharmacological treatments, as we had shown for furosemide treatment. For this set of experiments, we chose to test the effects of low-[CF]O treatment on spontaneous bursting which had been induced by high-[K+]O (12 mM) (n = 5), 4-AP (100 µM) (n = 4), and bicuculline (20 and 100 µM) (n = 5). In each set of experiments, field responses were recorded simultaneously from areas CA1 and CA3, and in each case, the spontaneous epileptiform activity in both areas CA1 and CA3, was reversibly blocked within 30 minutes after [CF]O in the perfusion medium had been reduced to 21 mM. These data suggest that, like furosemide, low-[CF]O reversibly blocks spontaneous bursting in several of the most commonly studied in vitro models of epileptiform activity.

EXAMPLE 110

Comparison between low-KTIQ and furosemide on blockade of high-[K+]O-induced epileptiform activity

The data from the previous sets of experiments are consistent with the hypothesis that the anti-epileptic effects of both low-[CF]O and furosemide are mediated by their actions on the same physiological mechanisms. To further test this hypothesis, we compared the temporal sequence of effects of low-[CF]O (n = 12) and furosemide (2.5 and 5 mM) (n = 4) on high-[K+]O-induced bursting, as recorded with a field electrode in CA1. We found that both low-[CF]O and furosemide treatment induced a similar temporal sequence of effects: an initial brief period of increased amplitude of field activity, and then blockade (reversible) of spontaneous field activity. In both cases, electrical stimulation of the Schaffer collaterals elicited hyperexcited responses even after the spontaneous bursting had been blocked.

EXAMPLE 111

Consequences of prolonged exposure to low-[0-)0 medium with varied fK+O

In the preceding experiments, we monitored field activity in some slices for > 1 hour after the spontaneous bursting had been blocked by low-[CF]O exposure. After such
prolonged low-[Cl\textsuperscript{-}]O exposure, spontaneous, long-lasting, depolarizing shifts developed. The morphology and frequency of these late-occurring field events appeared to be related to the extracellular potassium and chloride concentrations. Motivated by these observations, we performed a set of experiments in which we systematically varied [CF]O and [K\textsuperscript{+}]O and observed the effects of these ion changes on the late-occurring spontaneous field events.

In our first set of experiments, slices were exposed to medium containing low-[Cl\textsuperscript{-}]O (7 mM) and normal-[K\textsuperscript{+}]O (3 mM) (n = 6). After 50-70 minutes exposure to this medium, spontaneous events were recorded in area CAI; these events appeared as 5-10 mV negative shifts in the DC field, with the first episode lasting for 30-60 seconds. Each subsequent episode was longer than the previous one. This observation suggested that ion-homeostatic mechanisms were diminished over time as a result of the ion concentrations in the bathing medium. In some experiments (n = 2) in which these negative DC field shifts had been induced, intracellular recordings from CAI pyramidal cells were acquired simultaneously with the CAI field recordings.

For these experiments, the intracellular and field recordings were acquired close to one another (< 200 μm). Prior to each negative field shift (10-20 seconds), the neuron began to depolarize. Cellular depolarization was indicated by a decrease in resting membrane potential, an increase in spontaneous firing frequency, and a reduction of action potential amplitude. Coincident with the onset of the negative field shifts, the cells became sufficiently depolarized so that they were unable to fire spontaneous or current-elicited (not shown) action potentials. Since neuronal depolarization began 10-20 seconds prior to the field shift, it may be that a gradual increase in extracellular potassium resulted in the depolarization of a neuronal population, thus initiating these field events. Such an increase in [K\textsuperscript{+}]O might be due to alterations of the chloride-dependent glial cotransport mechanisms that normally move potassium from extracellular to intracellular spaces. To test whether increases in [K\textsuperscript{+}]O preceded these negative field shifts (and paralleled cellular depolarization), experiments (n=2) were performed in which a K\textsuperscript{+}-selective microelectrode was used to record changes in [K\textsuperscript{+}]O.

In each experiment, the K\textsuperscript{+}-selective microelectrode and a field electrode were placed in the CAI pyramidal layer close to one another (< 200 μm), and a stimulation pulse was delivered to the Schaffer collaterals every 20 seconds so that the magnitude of the population spike could be monitored. Multiple spontaneously occurring negative field
shifts were evoked by perfusion with low-[ClO] (7 mM) medium. Each event was associated with a significant increase in [K+]O, with the [K+]O increase starting several seconds prior to the onset of negative field shift. A slow 1.5-2.0 mM increase in [K+]O occurred over a time interval of approximately 1-2 minute seconds prior to the onset of each event. The stimulation-evoked field responses slowly increased in amplitude over time, along with the increasing [K+]O, until just before the negative field shift.

In a second set of experiments (n = 4), [K+]O was increased to 12 mM and [Cl-]O was increased to 16 mM. After 50-90 minutes exposure to this medium, slow oscillations were recorded in area CAI. These oscillations were characterized by 5-10 mV negative DC shifts in the field potential and had a periodicity of approximately 1 cycle/40 seconds. Initially, these oscillations occurred intermittently and had an irregular morphology. Over time, these oscillations became continuous and developed a regular waveform. Upon exposure to furosemide (2.5 mM), the amplitude of the oscillations was gradually decreased and the frequency increased until the oscillations were completely blocked. Such low-[Cl]O - induced oscillations in tissue slices have not been previously reported.

However, the temporal characteristics of the oscillatory events bear a striking resemblance to the low-[Cl]O - induced [K+]O oscillations which were previously described in a purely axonal preparation.

In a third set of experiments (n = 5) [Cl]O was further increased to 21 mM and [K+]O was reduced back to 3 mM. In these experiments, single, infrequently occurring negative shifts of the field potential developed within 40 - 70 minutes (data not shown). These events (5-10 mV) lasting 40-60 seconds, occurred at random intervals, and maintained a relatively constant duration throughout the experiment. These events could sometimes be elicited by a single electrical stimulus delivered to the Schaffer collaterals.

Finally, in a final set of experiments (n = 5), [Cl]O was kept at 21 mM and [K+]O was raised to 12 mM. In these experiments, late-occurring spontaneous field events were not observed during the course of the experiments (2-3 hours).

**EXAMPLE 112**

Changes in [K+]O during low-chloride exposure

Sprague-Dawley adult rats were prepared as previously described. Transverse hippocampal slices, 400 µm thick, were cut with a vibrating cuter and stored in an oxygenated holding chamber for 1 hour before recording. A submersion-type chamber
was used for K\textsuperscript{+}-selective microelectrode recordings. Slices were perfused with oxygenated (95% O\textsubscript{2}/5% CO\textsubscript{2}) artificial cerebrospinal fluid (ACSF) at 34-35°C. Normal ACSF contained 10 mM dextrose, 124 mM NaCl, 3 mM KCl, 1.25 mM Na\textsubscript{2}PO\textsubscript{4}, 1.2 mM MgSO\textsubscript{4}, 26 mM NaHCO\textsubscript{3} and 2 mM CaCl\textsubscript{2}. In some experiments, normal or low-chloride medium was used containing 4-aminopyridine (4-AP) at 100\textmu M. Low-chloride solutions (21 mM [Cl\textsubscript{0}]) were prepared by equimolar replacement of NaCl with Na\textsuperscript{+}-gluconate (Sigma Chemical Co.).

Field recordings from the CA1 or CA3 cell body layers were acquired with low-resistance glass electrodes filled with NaCl (2M). For stimulation of the Schaffer collateral pathway, a monopolar stainless-steel electrode was placed on the surface of the slice midway between areas CA1 and CA3. All recordings were digitized (Neurorocorder, Neurodata Instruments, New York, NY) and stored on videotape.

K\textsuperscript{+} selective microelectrodes were fabricated according to standard methods. Briefly, the reference barrel of a double-barreled pipette was filled with ACSF, and the other barrel was sylanized and the tip back-filled with KCl with K\textsuperscript{+}-selective resin (Coming 477317). Ion-selective microelectrodes were calibrated and considered suitable if they had a Nernstian slope response and remained stable throughout the duration of the experiment.

Exposure of hippocampal slices to low-[Cl\textsubscript{0}] medium has been shown to include a temporally-dependent sequence of changes on the activity of CA1 pyramidal cells, with three characteristics phases, as described above. In brief, exposure to low-[Cl\textsubscript{0}] medium results in a brief period of increased hyperexcitability and spontaneous epileptiform discharge. With further exposure to low-[Cl\textsubscript{0}] medium, spontaneous epileptiform activity is blocked, but cellular hyperexcitability remains, and action potential firing times become less synchronized with one another. Lastly, with prolonged exposure, the action potential firing times become sufficiently desynchronized so that stimulation-evoked field responses completely disappear, yet individual cells continue to show monosynapticllly-evoked responses to Schaffer collateral stimulation. The following results demonstrate that the antiepileptic effects of furosemide on chloride-cotransport antagonism are independent of direct actions on excitatory synaptic transmission, and are a consequence of a desynchronization of population activity with our any associated decrease in excitability.
In six hippocampal slices, K+-selective and field microelectrodes were placed in the CA1 cell body layer, and a stimulating electrode was placed on the Schaffer collateral pathway, and single-pulse stimuli (300 μs) were delivered every 20 seconds. After stable baseline [K+]o was observed for at least 20 minutes, the perfusion was switched to low-[Cl]o medium. Within 1-2 minutes of exposure to low-[Cl]o medium, the field responses became hyperexcitable as the [K+]o began to rise. After approximately 4-5 minutes of exposure to low-[Cr]o medium, the magnitude of the field response diminished until it was completely abolished. The corresponding recording of [K+]o showed that potassium began to rise immediately after exposure to low-[Cl]o medium, and that the peak of this [K+]o rise corresponded in time to the maximally hyperexcitable CA1 field response. Coincident with the reduction of the magnitude of the field response, the [K+]o began to diminish until after 8-10 minutes exposure to low-[Cr]o medium, it became constant for the remainder of the experiment at 1.8-2.5 mM above control levels. Four slices were switched back to control medium and allowed to fully recover. The experiment was then repeated with the K+-selective microelectrode placed in the stratum radiatum. A similar sequence of changes in [K+]o was observed in the dendritic layer, with the values of [K+]o being 0.2-0.3 mM less than those observed in the cell body layers.

In four hippocampal slices, the responses of stimulation-evoked changes in [K+]o between control conditions and after the CA1 field response was completely abolished by low-[Cl]o exposure were compared. In each slice, the [K+]o-selective measurements were acquired first in the cell body layer, and then after allowance for complete recovery in control medium, the experiment was repeated with the K+-selective electrode moved to the stratum radiatum. Each stimulation trial consisted of a 10 Hz volley delivered to the Schaffer collateral for 5 seconds. The peak rises in [K+]o were similar between control conditions an after prolonged exposure to low-[Cr]o medium, and between the cell body and dendritic layers. However, the recovery times observed after prolonged exposure to low-[Cl]o were significantly longer than those observed during control conditions.

These results demonstrate that the administration of furosemide resulted in increased [K+]o in the extracellular spaces. Exposure of the brain tissue to low-[Cl]o medium immediately induced a rise in [K+]o by 1-2 mM, which remained throughout the duration of exposure, and was coincident with the initial increase in excitability and the eventual abolishment of the CA1 field response. This loss of CA1 field response during low-[Cr]o exposure is most likely due to the desynchronization of neuronal firing times.
Significantly, the stimulation-evoked increases in \( [K^+ \text{J}_o \), in both the cell body and dendritic layers were nearly identical before and after the complete low-[Cl\(^-\)]_o blockade of the CA1 field response. This data suggests that comparable stimulation-evoked synaptic drive and action potential generation occurred under control conditions and after low \([\text{Cl}^-\text{]}_o\) blockade of the field. Together these data demonstrate that the antiepileptic and desynchronizing effects of the chloride-cotransport antagonist, furosemide, are independent of direct actions on excitatory synaptic transmission and are a consequence of a desynchronization of population activity without decrease in excitability.

**EXAMPLE 113**

**Changes in extracellular pH during low-chloride exposure**

Antagonists of the anion/chloride-dependent cotransporter, such as furosemide and low-[Cl\(^-\)]_o, may affect extracellular pH transients that might contribute to the maintenance of synchronized population activity. Rat hippocampal brain slices were prepared as described in Example 80, except the NaHCO\(_3\) was substituted by equimolar amount of HEPES (26 nM) and an interface-type chamber was used.

In four hippocampal brain slices continuous spontaneous bursting was elicited by exposure to medium containing 100 µM 4-AP, as described in Example 13. Field recordings were acquired simultaneously from the cell body layers in areas CA1 and CA3. A stimulus delivered every 30 seconds to the Schaffer collaterals throughout the duration of the experiments. After at least 20 minutes of continuous bursting was observed, the slices were exposed to nominally bicarbonate free, 4-AP-containing HEPES medium. There were no significant changes observed in the spontaneous or stimulation-evoked field responses resulting from prolonged exposure (0.2 hours) to HEPES medium. After the slices had been exposed for at least 2 hours to the HEPES medium, the perfusion was switched to 4-AP-containing HEPES medium in which the \([\text{Cl}^-\text{]}_o\) had been reduced to 21 mM. Exposure to the low-[Cr\(^-\)]_o HEPES medium induced the identical sequences of events, and at the same time course, as had previously been observed with low-[Cl\(^-\)]_o NaHCO\(_3\)-containing medium. After complete blockade of spontaneous bursting, the perfusion medium was switched back to HEPES medium with normal \([\text{Cr}^-\text{]}_o\). Within 20-40 minutes, spontaneous bursting resumed. At the time the spontaneous bursting had resumed, the slices had been perfused with nominally bicarbonate-free HEPES medium for greater than 3 hours.
This data suggests that the actions of chloride-cotransport antagonism on synchronization and excitability are independent of affects on the dynamics of extracellular pH.

Figure 4 illustrates a schematic model of ion cotransport under conditions of reduced [Cl\textsuperscript{−}]. Fig. 4A, left panel, shows that the chloride gradient necessary for the generation of IPSPs in neurons is maintained by efflux of ions through a furosemide-sensitive K\textsuperscript{+},Cl\textsuperscript{−} cotransporter. Under normal conditions, a high concentration of intracellular potassium (maintained by the 3Na\textsuperscript{+},2K\textsuperscript{+}-ATPase pump) serves as the driving force for the extrusion of Cl\textsuperscript{−} against its concentration gradient. In glial cells, as shown in the right panel of Fig. 4A, the movement of ions through the furosemide-sensitive NKCC co-transporter is from extracellular to intracellular spaces. The ion-gradients necessary for this cotransport are maintained, in part, by the "transmembrane sodium cycle": sodium ions taken into glial cells through NKCC cotransport are continuously extruded by the 3Na\textsuperscript{+},2K\textsuperscript{+}-ATPase pump so that a low intracellular sodium concentration is maintained. The rate and direction of ion-flux through the furosemide-dependent cotransporters are functionally proportional to their ion-product differences written as [K\textsuperscript{+}]\textsubscript{i} x [Cl\textsuperscript{−}]\textsubscript{o} - [K\textsuperscript{+}]\textsubscript{o} x [Cl\textsuperscript{−}]\textsubscript{i} for neuronal K\textsuperscript{+}, Cl\textsuperscript{−} cotransport and as [Na\textsuperscript{+}]\textsubscript{i} x [K\textsuperscript{+}]\textsubscript{i} x [Cl\textsuperscript{−}]\textsubscript{o} - [Na\textsuperscript{+}]\textsubscript{o} x [K\textsuperscript{+}]\textsubscript{o} x [Cl\textsuperscript{−}]\textsubscript{i} for glial NKCC cotransport. The sign of these ion-product differences show the direction of ion transport with positive being from intracellular to extracellular spaces.

Figure 4B shows a schematic phenomenological model that explains the emergence of the late-occurring spontaneous field events that arise as a result of prolonged low-[Cl\textsuperscript{−}]\textsubscript{o} exposure. We denote the ion-product differences for neurons and glia as QN and QG, respectively. Under control conditions (1), the differences of the ion-products for neurons are such that K\textsuperscript{+} and Cl\textsuperscript{−} are cotransported from intracellular to extracellular spaces (QN > 0); the differences in ion-products for glial cells are such that Na\textsuperscript{+}, K\textsuperscript{+} and Cl\textsuperscript{−} are cotransported from the ECS to intracellular compartments (QG < 0). When [Cl\textsuperscript{−}]\textsubscript{o} is reduced (2), the ion-product differences are altered so that neuronal efflux of KCl is increased; however, the glial icon cotransport is reversed (QG > 0), so that there is a net efflux of KCl and NaCl from intracellular to extracellular spaces. These changes result in buildup of extracellular potassium over time. Eventually, [K\textsuperscript{+}]\textsubscript{o} reaches a level that induces the depolarization of neuronal populations, resulting in an even larger accumulation of [K\textsuperscript{+}]\textsubscript{o}. This large accumulation of extracellular ions then serves to
reverse the ion-product differences so that KCl is moved from extracellular to intracellular spaces (QN < 0, QG < 0) (3). Further clearance of the extracellular potassium eventually resets the transmembrane ion gradients to initial conditions. By cycling through this process, repetitive negative field events are generated.

**EXAMPLE 114**

**Therapeutic Efficacy of Furosemide in the Alleviation of Pain Symptoms in an Animal Model of Neuropathic Pain**

The ability of furosemide to alleviate pain will be examined in rodents using the Chung model of neuropathic pain (see, for example, Walker et al. *Mol. Med. Today* 5:319-321, 1999). Sixteen adult male Long-Evans rats will be used in this study. All rats will receive spinal ligation of the L5 nerve as detailed below. Eight of the sixteen rats will receive an injection (intravenous) of furosemide and the remaining eight will receive intravenous injection of vehicle only. Pain threshold will be assessed immediately using the mechanical paw withdrawal test. Differences in pain thresholds between the two groups will be compared. If furosemide alleviates pain, the group with the furosemide treatment will exhibit a higher pain threshold than the group that received vehicle.

**Chung model of neuropathy**

Spinal nerve ligation is performed under isoflurane anesthesia with animals placed in the prone position to access the left L4-L6 spinal nerves. Under magnification, approximately one-third of the transverse process is removed. The L5 spinal nerve is identified and carefully dissected free from the adjacent L4 spinal nerve and then tightly ligated using a 6-0 silk suture. The wound is treated with an antiseptic solution, the muscle layer is sutured, and the incision is closed with wound clips. Behavioral testing of mechanical paw withdrawal threshold takes place within a 3 - 7 day period following the incision. Briefly, animals are placed within a Plexiglas chamber (20 x 10.5 x 40.5 cm) and allowed to habituate for 15 min. The chamber is positioned on top of a mesh screen so that mechanical stimuli can be administered to the plantar surface of both hindpaws. Mechanical threshold measurements for each hindpaw are obtained using an up/down method with eight von Frey monofilaments (5, 7, 13, 26, 43, 64, 106, and 202 mN). Each trial begins with a von Frey force of 13 mN delivered to the right hindpaw for approximately 1 sec, and then the left hindpaw. If there is no withdrawal response, the
next higher force is delivered. If there is a response, the next lower force is delivered. This procedure is performed until no response is made at the highest force (202 mN) or until four stimuli are administered following the initial response. The 50% paw withdrawal threshold for each paw is calculated using the following formula: \[ \text{[Xth]log} = \text{[vFrjlog} + \text{ky} \] where \([vFr]\) is the force of the last von Frey used, \(k = 0.2268\) which is the average interval (in log units) between the von Frey monofilaments, and \(y\) is a value that depends upon the pattern of withdrawal responses. If an animal does not respond to the highest von Frey hair (202 mN), then \(y = 1.00\) and the 50% mechanical paw withdrawal response for that paw is calculated to be 340.5 mN. Mechanical paw withdrawal threshold testing is performed three times and the 50% withdrawal values are averaged over the three trials to determine the mean mechanical paw withdrawal threshold for the right and left paw for each animal.

**EXAMPLE 115**

**Therapeutic Efficacy of Furosemide and Bumetanide in Alleviating the Symptoms of Intense Anxiety or Post Traumatic Stress Disorder**

The therapeutic usefulness of furosemide and bumetanide in the treatment of post traumatic stress disorder is examined by determining the ability of these compounds to alleviate intense anxiety in contextual fear conditioning in rats.

Contextual fear conditioning involves pairing an aversive event, in this case moderate foot shock, with a distinctive environment. The strength of the fear memory is assessed using freezing, a species-typical defensive reaction in rats, marked by complete immobility, except for breathing. If rats are placed into a distinctive environment and are immediately shocked they do not learn to fear the context. However, if they are allowed to explore the distinctive environment sometime before the immediate shock, they show intense anxiety and fear when placed back into the same environment. We can take advantage of this fact and, by procedurally dividing contextual fear conditioning into two phases, we can separately study effects of treatments on memory for the context (specifically a hippocampus based process) from learning the association between context and shock or experiencing the aversiveness of the shock (which depend upon emotional response circuitry including amygdala). Post traumatic stress syndrome (PTSD) in humans has been shown to be related to emotional response circuitry in the amygdala, and for this reason contextual memory conditioning is a widely accepted model for PTSD.
The experiment employed 24 rats. Each rat received a single 5 min episode of exploration of a small, novel environment. Seventy-two hours later they were placed into the same environment and immediately received two moderate foot-shocks (1 milliamp) separated by 53 sec. Twenty-four hours later, 8 of the rats received an injection (LV.) of furosemide (100 mg/kg) in vehicle (DMSO), and 8 of the rats were injected LV. with bumetanide (50 mg/kg) in vehicle (DMSO). The remaining 8 rats received an injection of DMSO alone. Each rat was again placed into the same environment for 8 min during which time freezing was measured, as an index of Pavlovian conditioned fear.

Four identical chambers (20 X 20 X 15 cm) were used. All aspects of the timing and control of events were under microcomputer control (MedPC, MedAssociates Inc., Vermont, USA). Measurement of freezing was accomplished through an overhead video camera connected to the microcomputer and was automatically scored using a specialty piece of software, FreezeFrame™ (OER Inc., Reston, VA). Total freezing time was analyzed in a one-way analysis of variance (ANOVA) test, with drug dose as the within-groups factor.

As shown in Fig. 5 significantly less freezing was observed in animals treated with either bumetanide or furosemide than in animals receiving vehicle alone, indicating that bumetanide and furosemide may be effectively employed in the treatment of post traumatic stress disorder.

**EXAMPLE 116**

**Therapeutic Efficacy of Furosemide and Bumetanide in Alleviating Anxiety**

The therapeutic efficacy of furosemide and bumetanide in alleviating anxiety was examined by evaluating the effects of these compounds in fear potentiated startle (FPS) test in rats. This test is commonly used to distinguish anxiolytic drug effects from non-specific effects, such as sedation/muscle relaxation.

Twenty-four rats received a 30 min period of habituation to the FPS apparatus. Twenty-four hours later, baseline startle amplitudes were collected. The rats were then divided into three matched groups based on baseline startle amplitudes. One of the rats exhibited a significantly higher baseline startle than the others and was excluded from the experiments. Groups 1 and 2 therefore consisted of 8 rats each, with Group 3 consisting of 7 rats. Following baseline startle amplitude collection, 20 light/shock pairings were delivered on two sessions over two consecutive days (i.e., 10 light/shock pairings per day). On the final day (day 5), Groups 2 and 3 received an injection (i.v.) of either
Furosemide (100 mg/kg) or bumetanide (70 mg/kg) in vehicle (DMSO) and Group 1 received vehicle alone. Immediately following injections, startle amplitudes were assessed during startle alone trials and startle plus fear (light followed by startle) trials. Fear potentiated startle (light + startle amplitudes minus startle alone amplitudes) was compared between the treatment groups.

Animals were trained and tested in four identical stabilimeter devices (Med-Associates). Briefly, each rat was placed in a small Plexiglas cylinder. The floor of each stabilimeter consisted of four 6-mm-diameter stainless steel bars spaced 18 mm apart through which shock can be delivered. Cylinder movements result in displacement of an accelerometer where the resultant voltage is proportional to the velocity of the cage displacement. Startle amplitude is defined as the maximum accelerometer voltage that occurs during the first 0.25 sec after the startle stimulus is delivered. The analog output of the accelerometer was amplified, digitized on a scale of 0-4096 units and stored on a microcomputer. Each stabilimeter was enclosed in a ventilated, light-, and sound-attenuating box. All sound level measurements were made with a Precision Sound Level Meter. The noise of a ventilating fan attached to a sidewall of each wooden box produced an overall background noise level of 64 dB. The startle stimulus was a 50 ms burst of white noise (5 ms rise-decay time) generated by a white noise generator. The visual conditioned stimulus employed was illumination of a light bulb adjacent to the white noise source. The unconditioned stimulus was a 0.6 mA foot shock with duration of 0.5 sec, generated by four constant-current shockers located outside the chamber. The presentation and sequencing of all stimuli were under the control of the microcomputer.

FPS procedures consisted of 5 days of testing; during days 1 and 2 baseline startle responses were collected, days 3 and 4 light/shock pairings were delivered, day 5 testing for fear potentiated startle was conducted.

Matching: On the first two days all rats were placed in the Plexiglas cylinders and 3 min later presented with 30 startle stimuli at a 30 sec interstimulus interval. An intensity of 105 dB was used. The mean startle amplitude across the 30 startle stimuli on the second day was used to assign rats into treatment groups with similar means.

Training: On the following 2 days, rats were placed in the Plexiglas cylinders. Each day following 3 min after entry, 10 CS-shock pairings were delivered. The shock was delivered during the last 0.5 sec of the 3.7 sec CSs at an average intertrial interval of 4 min (range, 3-5 min).
Testing: Rats were placed in the same startle boxes where they were trained and after 3 min were presented with 18 startle-eliciting stimuli (all at 105 dB). These initial startle stimuli were used to again habituate the rats to the acoustic startle stimuli. Thirty seconds after the last of these stimuli, each animal received 60 startle stimuli with half of the stimuli presented alone (startle alone trials) and the other half presented 3.2 sec after the onset of the 3.7 sec CS (CS-startle trials). All startle stimuli are presented at a mean 30 sec interstimulus interval, randomly varying between 20 and 40 sec.

Measures: The treatment groups were compared on the difference in startle amplitude between CS-startle and startle-alone trials (fear potentiation).

Fig. 6 shows the baseline startle amplitudes for each group of rats determined prior to the test day. Fig. 7 shows the amplitude of response on startle alone trials determined on the test day immediately following injection of either DMSO alone, bumetanide or furosemide, with Fig. 8 showing the difference score (startle alone - fear potentiated startle) on the test day. As shown in the figures, a statistically significantly lower difference score was observed in rats treated with either furosemide or bumetanide than in rats treated with vehicle alone, indicating that both furosemide and bumetanide are effective in reducing anxiety.

Figs. 9 and 10 show the startle alone amplitude and the difference score, respectively, one week after treatment with either furosemide or bumetanide. Animals treated with either furosemide or bumetanide were found to have a higher difference score than animals treated with vehicle alone. However, as the error bars are so large for the vehicle-treated animals, the data does not imply any statistically significant difference between vehicle and bumetanide, with possibly a small difference between vehicle and furosemide.

**EXAMPLE 117**

**Therapeutic Efficacy of Bumetanide Analogs in Alleviating Anxiety**

The therapeutic efficacy of several bumetanide analogs in alleviating anxiety was examined using the fear potentiated startle (FPS) test in rats as described above.

Fig. 11 shows the percent difference score (startle alone - fear potentiated startle) on the test day in rats treated with one of the following bumetanide analogs: bumetanide 3-(dimethylaminopropyl)ester; bumetanide benzytrimethylammonium salt; bumetanide dibenzylamide; bumetanide cyanomethyl ester; bumetanide N,N-diethylglycolamide ester; bumetanide N,N-dimethylglycolamide ester; bumetanide morpholinodethyl ester;
bumetanide pivaxetil ester; bumetanide methyl ester; bumetanide diethylamide; and bumetanide bezyl ester. The vehicle was DMSO. The number of animals tested for each bumetanide analog is shown in Table 1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>No. of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>34</td>
</tr>
<tr>
<td>Bumetanide</td>
<td>11</td>
</tr>
<tr>
<td>Bumetanide 3-(dimethylaminoproply) ester</td>
<td>17</td>
</tr>
<tr>
<td>Bumetanide benzylitrimethyl ammonium salt</td>
<td>12</td>
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<tr>
<td>Bumetanide dibenzyllamide</td>
<td>15</td>
</tr>
<tr>
<td>Bumetanide cyanomethyl ester</td>
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<tr>
<td>Bumetanide N,N-diethylglycolamide ester</td>
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</tr>
<tr>
<td>Bumetanide N,N-dimethylglycolamide ester</td>
<td>17</td>
</tr>
<tr>
<td>Bumetanide morpholinodethyl ester</td>
<td>17</td>
</tr>
<tr>
<td>Bumetanide pivaxetil ester</td>
<td>17</td>
</tr>
<tr>
<td>Bumetanide methyl ester</td>
<td>17</td>
</tr>
<tr>
<td>Bumetanide diethylamide</td>
<td>12</td>
</tr>
<tr>
<td>Bumetanide benzyl ester</td>
<td>12</td>
</tr>
</tbody>
</table>

As can be seen from Fig. 11, the percent difference score obtained after administration of most of the bumetanide analogs was significantly lower than that observed following administration of either vehicle alone or bumetanide, demonstrating that these analogs may be effectively employed to reduce anxiety. In addition, several of the bumetanide analogs were observed to have significantly lower diuretic effects than those generally associated with either furosemide or bumetanide.

While the present invention has been described with reference to the specific embodiments thereof, it will be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, method, method step or steps, for use in practicing the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

All patents and publications cited herein and PCT Application WO 00/37616, published June 29, 2000, are specifically incorporated by reference herein in their entireties.
SEQ ID NO: 1-2 are set out in the attached Sequence Listing. The codes for polynucleotide and polypeptide sequences used in the attached Sequence Listing conform to WIPO Standard ST.25 (1988), Appendix 2.
We claim:

1. A method for treating a disorder in a mammalian subject, comprising administering an effective amount of a composition comprising a Na⁺K⁺2Cl co-transporter antagonist to the subject, wherein the disorder is selected from the group consisting of: addictive disorders, and neuropsychiatric disorders.

2. The method of claim 1, wherein the Na⁺K⁺2Cl co-transporter antagonist reduces or blocks hypersynchronized neuronal population discharges by non-synaptic effects.

3. The method of claim 1, wherein the Na⁺K⁺2Cl co-transporter antagonist is a NKCC1 co-transporter antagonist.

4. The method of claim 1, wherein the Na⁺K⁺2Cl co-transporter antagonist is a CNS-targeted NKCC co-transporter antagonist.

5. The method of claim 4, wherein the Na⁺K⁺2Cl co-transporter antagonist is selected from the group consisting of: furosemide; bumetanide; ethacrynic acid; torsemide; azosemide; muzolimine; piretanide; tripamide; and functional analogs and derivatives thereof.

6. The method of claim 1, wherein the Na⁺K⁺2Cl co-transporter antagonist is selected from the group consisting of: thiazide; and thiazide-like diuretics.

7. The method of claim 1, wherein the Na⁺K⁺2Cl co-transporter antagonist is a compound selected from the group consisting of the following:

![Chemical Structure](image)
and

II,

III,

IV,

V

and

VI,
or a pharmaceutically acceptable salt, solvate, tautomer or hydrate thereof,
wherein
R₁ is not present, H, O or S;
R₂ is not present, H or when Ri is O or S, R₂ is selected from the group consisting of hydrogen, alkyl, aralkyl, aryl, alkylaminodialkyl, alkylcarbonylaminoc dialkyl, alkylxoxycarbonylalkyl, alkylcarbonyloxyalkyl, alkylaldehyde, alkylketoalkyl, alkylamide, alkarylamine, arylamine, an alkylammonium group, alkylcarboxylic acid, alkylheteroaryls, alkylhydroxy, a biocompatible polymer such as alkylxoxy(polyalkyloxy)alkylhydroxyl, a polyethylene glycol (PEG), a polyethylene glycol ester (PEG ester) and a polyethylene glycol ether (PEG ether), methoxyalkyl, methoxyalkaryl, methylthioalkyl and methylthioalkaryl, unsubstituted or substituted, and when Ri is not present, R₂ is selected from the group consisting of hydrogen, N₂N-dialkylamino, N,N-dialkarylamino, N,N-diarylamino, N-alkyl-N-alkarylamine, N-alkyl-N-arylamino, N-alkaryl-N-arylamino, unsubstituted or substituted;
R₃ is selected from the group consisting of aryl, halo, hydroxy, alkoxy, and aryloxy, unsubstituted or substituted; and
R₄ and R₅ are each independently selected from the group consisting of hydrogen, alkylaminodialkyl, carbonylalkyl, carbonylalkaryl, carbonylaryl and salts thereof, with the following provisos:
R₃ of formula I is not unsubstituted arylxoxy when R₁ is O and R₂, R₄ and R₅ are H;
R₃ of formula III is not Cl, when Ri is O and R₂, R₄ and R₅ are H;
R₁ of formula III is not methyl when Ri is O, R₃ is Cl, and R₄ and R₅ are H; and
R₃ of formula V is not unsubstituted arylxoxy when Ri is O and R₂, R₄ and R₅ are H.

8. The method of claim 1, wherein the compound is selected from the group consisting of bumetanide aldehyde, bumetanide methyl ester, bumetanide cyanomethyl ester, bumetanide ethyl ester, bumetanide isoamyl ester, bumetanide octyl ester, bumetanide benzyl ester, bumetanide dibenzylamine, bumetanide diethylamine, bumetanide morpholinoethyl ester, bumetanide 3-(dimethylamino propyl) ester, bumetanide N,N-diethylglycolamidoe ester, bumetanide dimethylglycolamidoe ester, bumetanide pivaxetil ester, bumetanide propaxetil ester, bumetanide methoxy(polyethyleneoxy)_n-i-ethyl ester, bumetanide benzytrimethylammonium salt, and bumetanide cetyltrimethylammonium salt.
9. The method of claim 7, wherein the compound is selected from the group consisting of bumetanide methyl thioester, bumetanide cyanomethyl thioester, bumetanide ethyl thioester, bumetanide isoamyl thioester, bumetanide octyl thioester, bumetanide benzyl thioester, bumetanide morphinoethyl thioester, bumetanide 3-(dimethylaminopropyl) thioester, bumetanide N,N-diethylglycolamide thioester, bumetanide dimethylglycolamide thioester, bumetanide pivaxetil thioester, bumetanide propaxetil thioester, bumetanide methoxy(polyethyleneoxy)$_{n-1}$-ethyl thioester, bumetanide thioacid (thiobumetanide), bumetanide benzyltrimethylammonium thioacid salt and bumetanide cetyltrimethylammonium thioacid salt.

10. The method of claim 7, wherein the compound is selected from the group consisting of metastable bumetanide thioacid, bumetanide O-methyl thioester, bumetanide O-cyanomethyl thioester, bumetanide O-ethyl thioester, bumetanide O-isoamyl thioester, bumetanide O-octyl thioester, bumetanide O-benzyl thioester, bumetanide O-(morphinoethyl) thioester, bumetanide O-[3-(dimethylaminopropyl)] thioester, bumetanide O-(N,N-diethylglycolamido) thioester, bumetanide, O-(dimethylglycolamido) thioester, bumetanide O-pivaxetil thioester, bumetanide O-propaxetil thioester, bumetanide O-[methoxy(polyethyleneoxy)$_{n-1}$-ethyl] thioester, bumetanide benzyltrimethylammonium thioacid salt and bumetanide cetyltrimethylammonium thioacid salt.

11. The method of claim 7, wherein the compound is selected from the group consisting of bumetanide thioaldehyde, bumetanide dithioacid, bumetanide methyl dithioester, bumetanide cyanomethyl dithioester, bumetanide ethyl dithioester, bumetanide isoamyl dithioester, bumetanide octyl dithioester, bumetanide benzyl dithioester, bumetanide dibenzylthioamide, bumetanide diethylthioamide, bumetanide morphinoethyl dithioester, bumetanide 3-(dimethylaminopropyl) dithioester, bumetanide N,N-diethylglycolamido dithioester, bumetanide dimethylglycolamido dithioester, bumetanide pivaxetil dithioester, bumetanide propaxetil dithioester, bumetanide methoxy(polyethyleneoxy)$_{n-1}$-ethyl dithioester, bumetanide benzyltrimethylammonium dithioacid salt and bumetanide cetyltrimethylammonium dithioacid salt.
12. The method of claim 7, wherein the compound is selected from the group consisting of furosemide methyl ester, furosemide cyanomethyl ester, furosemide ethyl ester, furosemide isoamyl ester, furosemide octyl ester, furosemide benzyl ester, furosemide morpholinoethyl ester, furosemide 3-(dimethylaminopropyl) ester, furosemide N,N-diethylglycolamido ester, furosemide dimethylglycolamido ester, furosemide pivaxetil ester, furosemide propaxetil ester, furosemide methoxy(polyethyleneoxy)$_n$-ethyl ester, furosemide benzytrimethylammonium acid salt and furosemide cetyltrimethylammonium acid salt.

13. The method of claim 7, wherein the compound is selected from the group consisting of furosemide thioacid, furosemide S-methyl thioester, furosemide S-cyanomethyl thioester, furosemide S-ethyl thioester, furosemide S-isoamyl thioester, furosemide S-octyl thioester, furosemide S-benzyl thioester, furosemide S-(morpholinoethyl) thioester, furosemide S-[3-(dimethylaminopropyl)] thioester, furosemide S-(N,N-diethylglycolamido) thioester, furosemide S-(diethylglycolamido) thioester, furosemide S-pivaxetil thioester, furosemide S-propaxetil thioester, furosemide S-[methoxy(polyethyleneoxy)$_n$-i-ethyl] thioester, furosemide benzytrimethylammonium thioacid salt, and furosemide cetyltrimethylammonium thioacid salt.

14. The method of claim 7, wherein the compound is selected from the group consisting of metastable furosemide thioacid, furosemide O-methyl thioester, furosemide O-cyanomethyl thioester, furosemide O-ethyl thioester, furosemide O-isoamyl thioester, furosemide O-octyl thioester, furosemide O-benzyl thioester, furosemide O-(morpholinoethyl) thioester, furosemide O-[3-(dimethylaminopropyl)] thioester, furosemide O-(N,N-diethylglycolamido) thioester, furosemide O-(dimethylglycolamido) thioester, furosemide O-pivaxetil thioester, furosemide O-propaxetil thioester, furosemide O-[methoxy(polyethyleneoxy)$_n$-i-ethyl] thioester, furosemide benzytrimethylammonium thioacid salt and furosemide cetyltrimethylammonium thioacid salt.

15. The method of claim 7, wherein the compound is selected from the group consisting of furosemide thioaldehyde, furosemide dithioacid, furosemide methyl dithioester, furosemide cyanomethyl dithioester, furosemide ethyl dithioester, furosemide isoamyl dithioester, furosemide octyl dithioester, furosemide benzyl dithioester, furosemide dibenzylthioamide, furosemide diethylthioamide, furosemide morpholinoethyl dithioester, furosemide 3-(dimethylaminopropyl) dithioester, furosemide
16. The method of claim 7, wherein the compound is selected from the group consisting of piretanide aldehyde, piretanide methyl ester, piretanide cyanomethyl ester, piretanide ethyl ester, piretanide isoamyl ester, piretanide octyl ester, piretanide benzyl ester, piretanide dibenzylamide, piretanide diethylamide, piretanide morpholinoethyl ester, piretanide 3-(dimethylaminopropyl) ester, piretanide N,N-diethylglycolamide ester, piretanide dimethylglycolamide ester, piretanide pivaxetil ester, piretanide propaxetil ester, piretanide methoxy(polyethyleneoxy)\textsubscript{n}-i-ethyl ester, piretanide benzyltrimethylammonium dithioacid salt and piretanide cetyltrimethylammonium dithioacid salt.

17. The method of claim 7, wherein the compound is selected from the group consisting of piretanide thioacid, piretanide S-methyl thioester, piretanide S-cyanomethyl thioester, piretanide S-ethyl thioester, piretanide S-isoamyl thioester, piretanide S-octyl thioester, piretanide S-benzyl thioester, piretanide S-(morpholinoethyl) thioester, piretanide S-[3-(dimethylaminopropyl)] thioester, piretanide S-(N,N-diethylglycolamido) thioester, piretanide S-(dimethylglycolarnido) thioester, piretanide S-pivaxetil thioester, piretanide S-propaxetil thioester, piretanide S-[methoxy(polyethyleneoxy)\textsubscript{n}-i-ethyl] thioester, piretanide benzyltrimethylammonium thioacid salt and piretanide cetyltrimethylammonium thioacid salt.

18. The method of claim 7, wherein the compound is selected from the group consisting of metastable piretanide thioacid, piretanide O-methyl thioester, piretanide O-cyanomethyl thioester, piretanide O-ethyl thioester, piretanide O-isoamyl thioester, piretanide O-octyl thioester, piretanide O-benzyl thioester, piretanide O-(morpholinoethyl) thioester, piretanide O-[3-(dimethylaminopropyl)] thioester, piretanide O-(N,N-diethylglycolamido) thioester, piretanide O-(dimethylglycolamido) thioester, piretanide O-pivaxetil thioester, piretanide O-propaxetil thioester, piretanide O-[methoxy(polyethyleneoxy)\textsubscript{n}-i-ethyl] thioester, piretanide benzyltrimethylammonium thioacid salt and piretanide cetyltrimethylammonium thioacid salt.
19. The method of claim 7, wherein the compound is selected from the group consisting of piretanide thioaldehyde, piretanide dithioacid, piretanide methyl dithioester, piretanide cyanomethyl dithioester, piretanide ethyl dithioester, piretanide isoamyl dithioester, piretanide octyl dithioester, piretanide benzyl dithioester, piretanide dibenzylthioamide, piretanide diethylthioamide, piretanide morpholinoethyl dithioester, piretanide 3-(dimethylaminopropyl) dithioester, piretanide N,N-diethylglycolamido dithioester, piretanide dimethylglycolamido dithioester, piretanide pivaxetil dithioester, piretanide propaxetil dithioester, piretanide methoxy(polyethyleneoxy)_{n} i-ethyl dithioester, piretanide benzyltrimethylammonium dithioacid salt and piretanide cetyltrimethylammonium dithioacid salt.

20. The method of claim 1, wherein the Na^{+}K^{+}2Cl co-transporter antagonist is a compound of formula VII:

![chemical structure](image)

or a pharmaceutically acceptable salt, solvate, tautomer or hydrate thereof,

wherein

R_{3} is selected from the group consisting of aryl, halo, hydroxy, alkoxy, and aryloxy, unsubstituted or substituted;

R_{4} and R_{5} are each independently selected from the group consisting of hydrogen, alkylaminodialkyl, carbonylalkyl, carbonylalkaryl, carbonylaryl and salts thereof;

R_{6} is selected from the group consisting of alkylxycarbonylalkyl, alkylaminocarbonylalkyl, alkylaminodialkyl, aikylhydroxy, a biocompatible polymer such as alkylxoy(polyalkyloxy)alkylhydroxyl, a polyethylene glycol (PEG), a polyethylene glycol ester (PEG ester) and a polyethylene glycol ether (PEG ether), methyloxyalkyl, methyloxyalkaryl, methylthioalkyl and methylthioalkaryl, unsubstituted or substituted, with the proviso that R_{3} is not Cl, when R_{4}, R_{5} and R_{6} are H.
21. The method of claim 20, wherein the compound is selected from the group consisting of tetrazolyl-substituted azosemide, azosemide benzyltrimethylammonium salt and azosemide cetyltrimethylammonium salt.

22. The method of claim 1, wherein the Na⁺K⁺2C¹ co-transporter antagonist is a compound of formula VIII:

![Chemical Structure](image)

or a pharmaceutically acceptable salt, solvate, tautomer or hydrate thereof, wherein

R₇ is selected from the group consisting of alkyloxy carbonylalkyl, alkylaminocarbonylalkyl, alkyldialkylalkyl, alkylhydroxy, a biocompatible polymer such as alkyloxy(polyalkoxy)alkylhydroxyl, a polyethylene glycol (PEG), a polyethylene glycol ester (PEG ester) and a polyethylene glycol ether (PEG ether), methyloxyalkyl, methyloxyalkaryl, methylthioalkyl and methylthioalkaryl, unsubstituted or substituted; and

X⁻ is a halide or an anionic moiety; or alternatively, X⁻ is not present.

23. The method of claim 22, wherein the compound is a pyridine-substituted torsemide quaternary ammonium salt.

24. The method of any one of claims 1-23, wherein the Na⁺K⁺2C¹ co-transporter antagonist modulates extracellular ion composition and chloride gradients in nervous system tissue.

25. The method of any one of claims 1-23, wherein the composition is delivered orally, sublingually, nasally, transdermally, intravenously, intrathecally, or by inhalation.
26. The method of any one of claims 1-25, wherein the disorder is a neuropsychiatric disorder selected from the group consisting of: bipolar disorders; anxiety disorders; depression; and schizophrenia.

27. The method of any one of claims 1-26, wherein the disorder is an anxiety disorder selected from the group consisting of: panic disorder; social anxiety disorder; obsessive compulsive disorder; posttraumatic stress disorder; generalized anxiety disorder; and specific phobia.

28. The method of any one of claims 1-25, wherein the disorder is an addictive disorder selected from the group consisting of: eating disorders; alcoholism; addiction to narcotics; and smoking.

29. The method of any one of claims 1-25 and 28, wherein the disorder is an eating disorder selected from the group consisting of: obesity; and binge eating.
**Fig. 1A**

CONTROL

2.5 mV
4 sec

25 msec
5 mV

**Fig. 1A1**
2.5 mM FUROSEMIDE

Fig. 1B
Glial

\[ \frac{[Na^+]_i}{[K^+]_i} < \frac{[Na^+]_o}{[K^+]_o} \]

\[ 3 Na^+ \rightarrow 2 K^+ \]

ECS

\[ 0 \]

Neuronal

\[ [K^+]_i > [K^+]_o \]

\[ [Cl^-]_i > [Cl^-]_o \]

\[ 3 Na^+ \rightarrow 2 K^+ \]
Fig. 4B
Contextual fear conditioning percent freezing

Fig. 5
**Fig. 6**

Pre test day - baseline startle amplitudes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Amplitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td></td>
</tr>
<tr>
<td>Bumetanide (70 mg/kg)</td>
<td></td>
</tr>
<tr>
<td>Furosemide (100 mg/kg)</td>
<td></td>
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</tbody>
</table>

**Fig. 7**

Test day - amplitude of response on startle alone trials

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Amplitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td></td>
</tr>
<tr>
<td>Bumetanide (70 mg/kg)</td>
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<td>Furosemide (100 mg/kg)</td>
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</tbody>
</table>
Fig. 8

Fig. 9
**Fig. 10**

FPS 1 week following compound testing

![Graph showing differences in FPS for previously vehicle, previously bumetanide, and previously furosemide treatments.](image)

**Fig. 11**

FPS - percent of controls

![Graph showing percent of controls for various compounds.](image)
SEQUENCE LISTING

<Hochman, Daryl W. Partridge, John J.>

Compositions And Methods For
The Treatment Of Neuropsychiatric
And Addictive Disorders

48000. 1005PCT
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12012 Human

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