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*[Continued on next page]*

(54) Title: HIGH THROUGHPUT BIOASSAY FOR IDENTIFYING SELECTIVE TRKA RECEPTOR AGONISTS, AND GAMBOGIC AMIDE, A SELECTIVE TRKA AGONIST WITH NEUROPROTECTIVE ACTIVITY

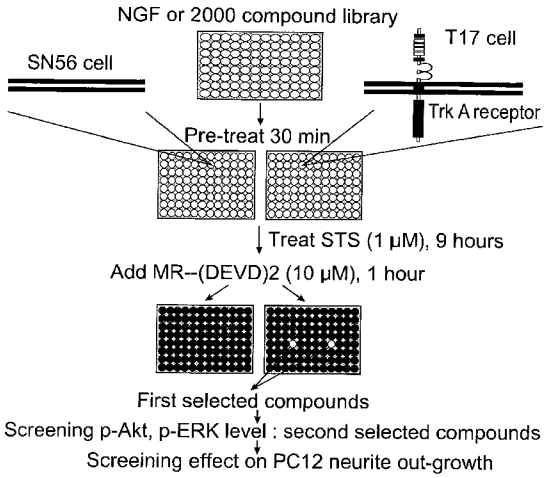


FIG.1A

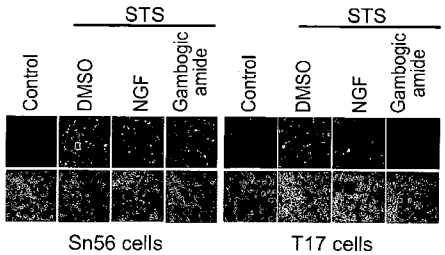


FIG.1B

(57) Abstract: A high-throughput screening assay for identifying small molecule agonists for the TrkA receptor is described. One such agonist, gambogic amide, selectively binds to TrkA but not TrkB or C, robustly induces its tyrosine phosphorylation and downstream signaling activation including Akt and MAP kinases. Further, it strongly prevents glutamate-induced neuronal cell death and provokes prominent neurite outgrowth in PC 12 cells. Gambogic amide specifically interacts with the cytoplasmic juxtamembrane domain of TrkA receptor and triggers its dimerization. Administration of this molecule in mice substantially diminishes Kainic acid-triggered neuronal cell death and decreases infarct volume in transient middle cerebral artery occlusion (MCAO) model of stroke. Thus, gambogic amide and other selective TrkA agonists can provide effective treatments for debilitating neurodegenerative diseases and provide neuroprotection from patients suffering from stroke or other ischemic events.

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# High Throughput Bioassay for Identifying Selective TrkA Receptor Agonists, and Gambogic Amide, a Selective TrkA Agonist with Neuroprotective Activity

## Cross-Reference to Related Applications

This application claims the benefit under 35 USC 119 of U.S. Provisional Patent Application No. 60/931,348 filed May 23, 2007. The disclosure of said U.S. Provisional Patent Application No. 60/931,348 is hereby incorporated herein by reference, in its respective entirety, for all purposes.

## Field of the Invention

The invention is generally in the area of high throughput bioassays for identifying selective TrkA agonists, compounds, such as Gambogic amide, identified using the bioassay, and methods of using the compounds to provide neuroprotection and/or treat or prevent neurodegenerative disorders.

## Background of the Invention

Neurotrophins play an essential role in the development and maintenance of the peripheral and the central nervous systems. The receptors for neurotrophins are members of a family of highly similar transmembrane tyrosine kinases (*Trk*A, *Trk*B and *Trk*C). Each neurotrophin binds to a preferred receptor in the family: nerve growth factor (NGF) binds mainly *Trk*A, brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4) bind *Trk*B and neurotrophin-3 binds *Trk*C, whereas p75NTR receptor non-selectively interacts with all members of the neurotrophins with similar affinity. Docking of TrkA by NGF initiates receptor dimerization, catalytic phosphorylation of cytoplasmic tyrosine residues on the receptor, and a cascade of cell-signaling events including activation of PI 3-kinase/Akt, Ras/Raf/MAP kinase and PLC-γ1 signaling pathways (Kaplan and Stephens, 1994). These signals lead to prevention of apoptotic cell death, promotion of cellular differentiation and axon elongation, and up-regulation of choline acetyl transferase (ChAT). Several neuronal cell types including sensory, sympathetic, and cholinergic neurons, implicated in various diseases, express TrkA and therefore respond to NGF (Mufson et al., 1997). In the normal adult human central nervous system (CNS), the cortical and nucleus

basalis neurons that comprise the cholinergic system play essential roles in learning and memory.

In neurodegenerative processes, such as mild cognitive impairment (MCI), loss of TrkA density correlates with neuronal atrophy and precedes neuronal death and severe cognitive impairment in Alzheimer's Disease (AD) (Counts et al., 2004). In MCI-AD progression, loss of TrkA correlates with cognitive decline. In basal forebrain neurons of aged rats, the expression of NGF receptors is decreased, but can be reversed by NGF administration (Backman et al., 1997). It has been suggested that NGF therapy may delay the onset of Alzheimer's disease (Barinaga, 1994; Lindsay, 1996) and ameliorate peripheral diabetic neuropathies (Ebadi et al., 1997). Other applications proposed for NGF include treatment of neuronal damage (Hughes et al., 1997) and targeting of neuroectoderm-derived tumors (Cortazzo et al., 1996; LeSauteur et al., 1995). Both *in vitro* and in animal models studies demonstrate that NGF might be clinically useful for treating CNS diseases (Connor and Dragunow, 1998). As expected, preclinical and clinical findings also suggest that subcutaneous or intravenous administration of neurotrophins may be an effective treatment for peripheral neurodegenerative disorders (McArthur et al., 2000; McMahon and Priestley, 1995).

Despite the therapeutic potential of NGF, clinical trials featuring this protein have been disappointing (Verrall, 1994). There are several reasons for this, including the inherent drawbacks associated with using polypeptides as drugs (Saragovi et al., 1992), *in vivo* instability (Barinaga, 1994), and pleiotrophic effects due to activation of signals that were not intentionally targeted (e.g., those mediated via the low-affinity NGF receptor p75) (Carter and Lewin, 1997). Moreover, NGF protein is relatively expensive to produce for medicinal applications. In order to circumvent the above curbs, substantial efforts have been made to design small, proteolytically stable molecules with neurotrophic activity, selective for cells expressing TrkA (Lee and Chao, 2001; Saragovi et al., 1991; Saragovi et al., 1992). They present partial agonistic activity in the absence of NGF by activating TrkA. Although they do not directly dimerize the receptor, the compounds cause conformational changes on TrkA that stabilize its dimerization. Thus, they act more as potentiators of NGF action rather than as robust TrkA agonists.

It would be advantageous to provide high-throughput bioassays to identify compounds that are selective TrkA agonists, and selective TrkA agonists, and methods. The present invention provides such bioassays.

## Summary of the Invention

A high-throughput cell-based chemical genetic screen using caspase-3 activated fluorescent dye as read-out is disclosed. Numerous identified compounds selectively protect TrkA-expressed cells, but not the parental clone without the receptor.

The screen involves a cell-based apoptotic assay that compares the ability of a test compound with the ability of nerve growth factor (NGF) to protect cells expressing TrkA from apoptosis. The assay uses a cell permeable fluorescent dye, such as MR(DERD)2, which turns red upon caspase-3 cleavage in apoptotic cells. The cells used in the assay are from a murine cell line T17, which was derived from basal forebrain SN56 cells. T17 cells are TrkA stably transfected SN56 cells.

Compounds that protect these cells from apoptosis are either TrkA agonists or partial agonists, or affect downstream processes. To help ascertain whether the compounds act on TrkA, or on a downstream step, one can look at whether the compounds cause TrkA to dimerize. That is, compounds which cause TrkA to dimerize act on TrkA, not on a downstream step. One way to determine whether test compounds can trigger TrkA dimerization is to co-transfect GFP-TrkA and HA-TrkA into HEK293 cells, expose the co-transfected cells to the compounds.

Those compounds which inhibit apoptosis and cause TrkA to dimerize are TrkA agonists (or partial agonists). To determine whether the compounds are selective TrkA agonists (i.e., bind selectively to TrkA in preference to TrkB or TrkC), one can use cells that are cotransfected with TrkB and TrkA receptors or TrkC and TrkA receptors. If the compounds are selective for TrkA, the receptors will fail to dimerize. Selective TrkA agonists will also elicit tyrosine phosphorylation in TrkA, but not in TrkB or C receptors (or in cells co-transfected to express two or more of these receptors, i.e., TrkA in combination with TrkB and/or TrkC). So, one can look for tyrosine phosphorylation as well to determine whether the compounds are selective TrkA agonists or partial agonists.

Once compounds with desired properties are identified, their actual activity can be assessed, for example, by determining whether the compounds provoke neurite

outgrowth in PC12 cells, and/or prevent neuronal cell death. Compounds with one or both of these activities can be useful compounds for treating neurodegenerative disorders and/or providing neuroprotection.

One class of such compounds, gambogic acid and certain analogues thereof, bind with relatively high affinity to TrkA. Representative gambogic acid analogues, and their preparation, are described in U.S. Patent No. 6,462,041, U.S. Patent No. 6,613,762, and U.S. Patent No. 7,176,234, the contents of each of which are hereby incorporated by reference in their entirety. These compounds can be prepared and screened for their ability to either cause apoptosis (in which case they may be active anti-cancer agents) or inhibit apoptosis (in which case they may be selective TrkA agonists, and therefore useful, for example, as neuroprotective compounds), using the assays described herein.

Gambogic amide was identified as a compound which selectively binds to TrkA, triggers its tyrosine phosphorylation, elicits PI 3-kinase/Akt and MAP Kinase activation, provokes neurite outgrowth in PC12 cells, and prevents neuronal cell death. Moreover, it substantially decreases the infarct volume following MCAO. Thus, gambogic amide mimics NGF and possesses potent neurotrophic activities.

The selective TrkA agonists can be used to provide neuroprotection, before, during, or after a neural insult, such as a stroke or other ischemic event. The compounds can also be used to to treat demyelinating diseases such as multiple sclerosis (MS), to enhance nerve regeneration, and/or to treat or prevent neurodegenerative diseases. Such neurodegenerative diseases include, but are not limited to, epilepsy, head and spinal chord trauma, Parkinson disease, Huntington's disease, Alzheimer's disease, or amyotrophic lateral schlerosis, or a neurological disorder.

The screening method, selective TrkA agonists or partial agonists, and methods of providing neuroprotection, or treating and/or preventing neurodegenerative diseases or demyelinating diseases, will be better understood with reference to the detailed description below.

**Brief Description of the Figures**

Figure 1A is a schematic flow chart showing one embodiment of the TrkA agonist screening method described herein.

Figure 1B shows representative microscopic pictures from SN56 and T17 cells pretreated with NGF or gambogic amide, followed by STS. The apoptotic cells were stained with red fluorescent activated by caspase-3.

Figure 2A shows the chemical structures of various gambogic acid derivatives (left panel), and the chemical structure of gambogic amide, with the numeric positions labeled (right panel).

Figure 2B is a graph showing that gambogic acid derivatives prevent apoptosis in T17 cells, but not SN56 cells.

Figure 2C is a graph showing that gambogic amide exhibits the strongest anti-apoptotitc activity in primary hippocampal neurons (left panel). As shown in the graph, the $EC_{50}$ for gambogic acid derivatives in preventing apoptosis in T17 cells are: 10 nM for gambogic amide, 50 nM for dihydrogambogic acid, 55 nM for gambogic acid, 750 nM for dimethyl gambogate (right panel).

Figure 2D is a graph showing that gambogic amide protects hippocampal neurons from glutamate-triggered apoptosis.

Figure 2E is a graph showing that gambogic amide protects neurons from apoptosis triggered by OGD (left panel). As shown in the graph, gambogic amide displayed a dose-dependent protective manner on neurons in OGD (right panel).

Figure 3A is a photograph of PC12 cells treated with NGF or 0.5 μM gambogic acid derivatives for 5 days. As shown in the photograph, gambogic amide triggered neurite outgrowth as potently as NGF, and dihydrogambogic acid also displayed weak stimulatory activity. By contrast, other derivatives had no effect.

Figure 3B is a photograph showing the dose-dependent effect of neurite outgrowth. 10-50 nM gambogic amide can provoke neurite outgrowth in PC12 cells.

Figure 4 are photographs showing that gambogic amide elicits TrkA tyrosine phosphorylation in hippocampal neurons.

Figure 4A shows an immunoblotting analysis with anti-phospho-Trk490 of hippocampal neurons, treated with 0.5 μM gambogic acid derivatives for 30 min. The analysis shows that gambogic acid derivatives induce TrkA tyrosine phosphorylation in primary neurons.

Figure 4B is a photograph of the immunofluorescent staining of gambogic acid derivative-treated hippocampal neurons with anti-phospho-Trk490 antibody. As shown in the photograph, both NGF and gambogic amide selectively triggered TrkA Y490 phosphorylation in neurons.

Figure 4C is a photograph showing that gambogic amide provokes TrkA dimerization. GFP-TrkA and HA-TrkA or HA-TrkB were respectively cotransfected into HEK293 cells, and treated with 0.5 μM gambogic acid or amide for 30 min. GFP-TrkA was immunoprecipitated with anti-GFP antibody, and the coprecipitated proteins were analyzed with anti-HA antibody. Gambogic amide, but not gambogic acid, evidently stimulated TrkA dimerization. The stimulatory effect was even stronger than NGF; by contrast, TrkA did not bind to TrkB regardless of NGF or gambogic amide treatment (top panel).

Figure 4D is a photograph showing that gambogic amide triggered TrkA but not TrkB or C tyrosine phosphorylation in transfected HEK293 cells, and that kinase-dead TrkA displayed decreased tyrosine phosphorylation.

Figure 5 is a photograph showing that gambogic amide provokes MAP Kinase and Akt kinase activation.

Figure 5A shows that gambogic amide selectively provokes Erk phosphorylation in T17 cells, whereas other derivatives exhibited weakly stimulatory effects. By contrast, in addition to gambogic amide, gambogic acid and decahydrogambogic acid also triggered robust Akt activation.

Figure 5B shows that gambogic acid derivatives initiate Akt activation in hippocampal neurons.

Figure 5C shows a characterization of gambogic amide's stimulatory effect on Akt phosphorylation. As shown in the photograph, gambogic amide elicited Akt activation in a time-dependent manner (left), and induced Akt activation in a dose-dependent manner (right panel).

Figure 6 shows that the juxtamembrane domain of TrkA binds gambogic acid.

Figure 6A shows the expression of transfected various GFP-TrkA mutants by immunoblotting with anti-GFP antibody (top panels). As shown in the immunoblotting, some of the intracellular domain truncated TrkA mutants including the whole intracellular domain (ΔICD) and SHC binding domain deleted (ΔSHC)

mutants failed to bind gambogic acid (lower panels). This data shows that the extracellular domain truncated TrkA mutants potently bind to gambogic amide.

Figure 6B shows a sequence alignment of the ICD of TrkA, B and C.

Figure 6C shows that gambogic amide selectively bound to TrkA but not B or C receptor. The tyrosine kinase-dead TrkA decreased its binding affinity to gambogic amide (upper panel). The expression of transfected constructs was verified (lower panel).

Figure 6D shows a competition assay for the dissociation constant of gambogic amide (relative binding amount/nM concentration of gambogic amide). GFP-TrkA-associated gambogic amide-beads were incubated with increased gambogic amide concentrations. After extensive washing, the beads bound proteins were monitored by immunoblotting and quantitated by NIH Image software. The data show that the $K_d$ for gambogic amide to TrkA is about 75 nM. The inactive control tetrahydrogambogic acid did not compete for binding at all.

Figure 6E are photographs showing that FITC-Gambogic amide penetrates cell membrane and binds to TrkA receptor. PC12 cells were incubated for 10 min with 0.5 μM FLTC-gambogic amide and control FITC, respectively. After washing and fixing, the cells were stained with TrkA antibody.

Figure 7 shows that gambogic amide prevents neuronal cell death and decreases infarct volume in MCAO animal model.

Figure 7A shows brain slides analyzed with a TUNEL assay and stained with DAPI. The data show that gambogic amide diminished Kainic acid-triggered hippocampal neuronal cell death. The TUNEL slides show apoptotic nuclei, which were also stained with DAPI. Kainic acid evidently initiated devastating apoptosis in hippocampal CA3 region, which was substantially blocked by gambogic amide (left panel). Quantitative analysis of apoptosis in the hippocampal neurons (right panel).

Figure 7B is a chart showing that gambogic amide reduces infarct volume in MCAO rat brain. TTC-stained coronal section from representative animals given either vehicle or Gambogic amide and had brains harvested at 24h post occlusion. Infarcts are shown as pale (unstained) regions involving striatum and overlying cortex. The infarct area in gambogic amide-treated animals is substantially reduced (top panel).† Infarct volumes after 24 h MCAO. Compared to vehicle alone, gambogic amide significantly reduced total infarct volumes (% of contralateral

hemisphere). The data are represented as mean ± SD; †* ($p<0.05$) = significant difference compared to MCAO + Vehicle (middle panel). Laser-Doppler measurement of relative CBF during 2 hours of MCAO and 5min post-reperfusion. LDF was measured over the ipsilateral parietal cortex and expressed as a percentage of baseline. There were no significant differences between groups, suggesting that the relative ischemic insult was equivalent among all groups (bottom panel).

## Detailed Description

High throughput screening methods for identifying selective TrkA agonists, selective agonists, such as gambogic amides, identified using the screening methods, and methods of treatment and prevention using the compounds, are disclosed.

The present invention arises out of the discovery that novel derivatives and analogs of gambogic acid are also selective TrkA agonists. Therefore, these derivatives and analogs of gambogic acid are potentially useful for providing neuroprotection, treating demyelinating disorders, and for treating and/or preventing neurodegenerative disorders.

### I. High Throughput Screening Method

The following high-throughput screening assay was developed to identify small molecules that mimic NGF and activate TrkA, ideally in a selective manner. That is, the assay can identify compounds that are agonists or partial agonists for TrkA, and ideally those which are selective for TrkA over TrkB and TrkC.

The cell-based apoptotic assay uses a caspase assay system, which include fluorometric, colorimetric, cell proliferation and cell viability assay systems, which include a fluorometric or colorimetric marker for apoptotic cells. In one embodiment, the cell-based apoptotic assay uses a cell permeable fluorescent dye, such as MR(DERD)2, which turns red upon caspase-3 cleavage in apoptotic cells. The cells used in the assay can be from any cell line that does not express TrkA. In one embodiment, the cells are from a murine cell line T17, which was derived from basal forebrain SN56 cells. T17 cells are TrkA stably transfected SN56 cells.

Those candidate compounds that selectively protect T17, but not SN56 cells, are either TrkA agonists, or function by activating a downstream pathway. Even if they are TrkA agonists, it is not clear from the anti-apoptotic activity alone that they are selective TrkA agonists. So, additional screening steps can be performed to

determine whether the compounds are TrkA agonists, and if so, whether they are selective TrkA agonists.

To help ascertain whether the compounds act on TrkA, or on a downstream step, one can look at whether the compounds cause TrkA to dimerize. That is, compounds which cause TrkA to dimerize act on TrkA, not on a downstream step. One way to determine whether test compounds can trigger TrkA dimerization is to co-transfect GFP-TrkA and HA-TrkA into HEK293 cells, expose the co-transfected cells to the compounds.

As used herein, a TrkA agonist is a compound with at least 90% of the activity of NGF in selectively protecting T17, but not SN56 cells, and which causes TrkA to dimerize, and a partial agonist is a compound with less than 90% of the activity of NGF in the selective protection of these cells, and which causes TrkA to dimerize. A selective TrkA agonist (or partial agonist) is a compound which exhibits substantial binding affinity to TrkA over TrkB and/or TrkC, for example, at least 4:1 binding affinity for TrkA over TrkB and/or TrkC.

Those compounds which inhibit apoptosis and cause TrkA to dimerize are TrkA agonists (or partial agonists). To determine whether the compounds are selective TrkA agonists (i.e., bind selectively to TrkA in preference to TrkB or TrkC), one can use cells that are cotransfected with TrkB and TrkA receptors or TrkC and TrkA receptors. If the compounds are selective for TrkA, the receptors will fail to dimerize. Selective TrkA agonists will also elicit tyrosine phosphorylation in TrkA, but not in TrkB or C receptors (or in cells co-transfected to express two or more of these receptors, i.e., TrkA in combination with TrkB and/or TrkC). So, one can alternatively, or also, look for tyrosine phosphorylation to determine whether the compounds are selective TrkA agonists (or partial agonists). Dose studies can be performed to determine whether the compounds are agonists or partial agonists.

Once compounds with desired properties are identified, their actual activity can be assessed, for example, by determining whether the compounds provoke neurite outgrowth in PC12 cells, and/or prevent neuronal cell death. Compounds with one or both of these activities can be useful compounds for treating neurodegenerative disorders and/or providing neuroprotection.

Ideally, compounds testing positive in both of these screens can be analyzed for TrkA tyrosine phosphorylation, and/or Akt and MAP kinases signaling cascade

activation. The screening strategy scheme is depicted in Figure 1A, and each of the assay steps is described in more detail below.

T17 cells can be cultured in multi-well plates, such as 96-well plates, and preincubated with around 10 μM of putative TrkA agonists for a period of time, for example, 30 min, followed by 1 μM staurosporine (STS) treatment for a period of time, for example, 9 h. When the dye is MR(DEVD)2, and this dye is introduced to the cells (for example, 1 h before examination under a fluorescent microscope), apoptotic cells will appear red, while live cells will have no signal. NGF can be used as a positive control. NGF will substantially decrease the red cell numbers compared to a control, such as a DMSO control.

As discussed below in the Examples section, one embodiment of the assay using the caspase-3-activated fluorescent dye, 2000 biologically active compounds from the Spectrum Collection Library were screened. The screen identified thirty-one compounds which selectively protected T17, but not SN56 cells, from STS-initiated apoptosis. This indicated that the compounds might act either directly through the TrkA receptor, or its downstream signaling effectors.

Even in the absence of NGF, T17 exhibits a stronger anti-apoptotic effect than its parental SN56 cells, indicating that overexpression of TrkA suppresses caspase-3 activation. NGF treatment further enhances this effect (Figure 1B, right panel).

**Identification of Compounds as Survival Enhancers**

To compare the apoptosis inhibitory activity of compounds identified in the first screen, compounds can be pre-incubated (0.5 μM) with T17 and SN56 cells, followed by 1 μM STS for 9 h. Quantitative analysis of the apoptosis inhibitory activities will reveal which compounds have little or no ability to protect SN56 cells from apoptosis, but which strongly suppress apoptosis in T17 cells ideally with protective activities even stronger than NGF). Such compounds may show use in neuroprotection.

Compounds identified in the screen that increase apoptosis in T17 cells can trigger programmed cell death, and may be useful as anti-cancer agents.

TrkA is highly expressed in hippocampal neurons (Culmsee et al., 2002; Kume et al., 2000; Zhang et al., 1993). TrkA and p75NTR are up-regulated in hippocampal and cortical neurons under pathophysiological conditions (Kokaia et al.,

1998; Lee et al., 1998). Moreover, neuroprotective effects of NGF in hippocampal and cortical neurons have been demonstrated *in vitro* and *in vivo* (Culmsee et al., 1999; Zhang et al., 1993).

To examine whether test compounds can promote neuronal survival, hippocampal neurons can be prepared, and the primary neurons pre-treated with various test compounds for a period of time, for example, 30 min, followed by 50 μM glutamate treatment for a period of time, for example, 16 h. A quantitative apoptosis assay with MR(DEVD)2 can be used to determine whether the compounds display a comparable protective effect as the positive control NGF (i.e., apoptosis inhibitory activity).

NGF overexpression decreases infarct volume and neuronal apoptosis in transgenic mice or intraventricular injected mice (Guegan et al., 1998; Luk et al., 2004). NGF also potently protects PC12 cells from apoptosis in an OGD (Oxygen-glucose-deprivation) model (Tabakman et al., 2005). To explore whether test compounds might exert any protective effect on hippocampal neurons in OGD, the primary preparations can be pre-treated with NGF or test compounds 30 min before OGD stimulation. In 3 h, apoptotic analysis can be used to show whether the compounds exhibit potent protective effects. Titration assays can show whether the compounds protect neurons in a dose-dependent manner. Therefore, the assay can identify compounds which selectively protect TrkA expression cells and primary neurons from apoptosis.

**Determination of Neurite Outgrowth in PC12 cells**

One of most prominent neurotrophic effects of NGF is to trigger neurite outgrowth in neuronal cells and incur differentiation. To explore whether test compounds possess this activity, PC12 cells can be incubated with a certain concentration of test compounds (for example, 0.5 μM of the compounds) for a period of time, such as 5 days. The cell medium containing the compounds can be replenished periodically, such as every other day.

In this type of assay, NGF will elicit pronounced neurite sprouting in PC12 cells after 5 days of treatment. Those test compounds which are active in one or more of the assays described above, and which also elicit neurite outgrowth in PC12 cells, can be identified as neurotrophic/neuroprotective.

The neurite network generated by a test compound can be used as evidence that the compounds have strong neurotrophic activity. Dose-dependent assays can be used to reveal ideal concentrations to provoke substantial neurite sprouting in PC12 cells, and thus identify compounds which possess potent neurotrophic activity at a concentration comparable to NGF, and robustly provoke neurite outgrowth.

**Identification of Compounds Which Trigger TrkA tyrosine phosphorylation in hippocampal neurons**

NGF binds to receptor TrkA and elicits its dimerization and autophosphorylation on tyrosine residues. Numerous tyrosine residues on TrkA are phosphorylated upon NGF stimulation. For example, Y490 phosphorylation is required for Shc association and activation of MAP kinase signaling cascade. Y751 phosphorylation is essential for PI 3-kinase docking and activation.

To evaluate whether test compounds can also trigger TrkA tyrosine phosphorylation, primary hippocampal neurons can be treated with putative compounds at a certain concentration (for example, 0.5 μM), for a certain period of time (for example, 30 min.). The cell lysates can be analyzed, for example, by immunoblotting with anti-phospho-TrkA Y490 antibody.

NGF treatment will be shown to induce potent TrkA phosphorylation, and the assay can identify compounds which, like NGF, similarly induce TrkA phosphorylation. TrkA tyrosine phosphorylation in hippocampal neurons can also be demonstrated by immunofluorescent staining with an anti-TrkA Y490 specific antibody.

To determine whether test compounds can trigger TrkA dimerization, GFP-TrkA and HA-TrkA can be cotransfected into HEK293 cells, and the cells treated with 0.5 μM gambogic amide for 30 min. Coimmunoprecipitation assays can be used to determine whether any test compounds provoke TrkA dimerization, ideally even more strongly than NGF, more ideally with a negative control such as DMSO to generate a baseline.

Cotransfected TrkA and TrkB or TrkC receptors will fail to dimerize regardless of pre-treatment with NGF or with compounds that also selectively trigger dimerization of the TrkA receptors. Selective TrkA agonists will elicit tyrosine phosphorylation in TrkA, but not in TrkB or C receptors (or in cells co-transfected to

express two or more of these receptors, i.e., TrkA in combination with TrkB and/or TrkC).

Some compounds may be effected in such a manner that TrkA-KD will display decreased tyrosine phosphorylation compared to wild-type TrkA, which will indicate that not only TrkA autophosphorylation but also other tyrosine kinases are activated, and contribute to Y490 phosphorylation. These assays can thus identify compounds which mimic NGF and selectively provoke TrkA dimerization and tyrosine phosphorylation.

**Identification of Compounds Which Provokes Akt and MAP kinase activation**

NGF triggers PI 3-kinase/Akt and Ras/MAP Kinase signaling cascades activation through the TrkA receptor. To explore whether test compounds possess similar mitogenic effects, T17 cells can be treated with various test compounds for 30 min. The cell lysates can be analyzed by immunoblotting with anti-phospho-Erk1/2 and phospho-Akt-473 antibodies, respectively.

NGF treatment stimulates demonstrable Erk1/2 and Akt phosphorylation. Those compounds which similarly provoke robust phosphorylation of both Erk1/2 and Akt will be shown to be effective TrkA agonists (or partial agonists).

Compounds which are unable to activate MAP kinase, but which strongly provoke Akt activation, might differentially regulate PI 3-kinase/Akt and Ras/MAP kinase pathways either through the TrkA receptor or its downstream cellular targets.

The assays can be extended into primary hippocampal neurons. NGF and certain test compounds may activate Akt, while other compounds may slightly upregulate Akt phosphorylation. Time course assays with hippocampal neurons can be used to show that certain compounds elicit Akt phosphorylation after 5 min treatment, and whether Akt activation increases over time, whether it can be sustained, and whether it provokes Akt activation in a dose-dependent manner. Taken together, this information can identify compounds which mimic NGF and potently activate Akt and MAP kinase activation in neurons.

**Identification of Compounds Which Bind the Cytoplasmic Juxtamembrane Domain of TrkA receptor**

The immunoglobulin (Ig)-like domain (TrkA-d5 domain) in the extracellular region of TrkA proximal to the membrane is required for specific binding of NGF (Urfer et al., 1995). To investigate which portion of TrkA receptor binds to test compounds that also appear to be TrkA agonists, based on the assays described above, *in vitro* binding assay can be conducted with immobilized compounds by covalently linking the compounds to a solid substrate, such as affi-gel 102.

GFP-tagged TrkA truncates can be prepared, and transfected into HEK293 cells. Binding assays can be used to show whether the extracellular domain is or is not required for the association. The cytoplasmic juxtamembrane domain is critical for ligand binding. Although the intracellular domain (ICD) of Trk family members shares great homology, the juxtamembrane region varies. *In vitro* binding assay can be used to show whether test compounds selectively bound to wild-type and/or kinase-dead TrkA, but not to TrkB or TrkC. Thus, selective TrkA agonists (or partial agonists) can be identified.

If test compounds have a weaker affinity to Trk-KD than to wild-type TrkA, and/or fail to bind to p75NTR, ErbB3 or EGF receptors, this can show that the test compounds specifically associate with TrkA but not other neurotrophin receptors or transmembrane tyrosine kinase receptors.

To determine the binding constant between test compounds and TrkA, one can conduct a competition assay with GFP-TrkA-bound beads covalently linked to the test compounds. If the concentration of bead-associated TrkA is gradually decreased as the free concentration of test compounds increases, quantitative analysis of the competition data will show the $K_d$ of the test compounds. Incubation of FITC-conjugated test compounds with PC12 cells for 10 minutes can elicit their association with TrkA receptors, whereas FITC alone fails to penetrate into cells. Using this information, one can determine whether a test compound penetrates the cell membrane and binds tightly to TrkA receptor through the same or a different region than NGF.

**Determination of whether a Test Compound Can Prevent Kainic acid-Triggered Neuronal Apoptosis and Decreases Infarct Volume in Stroked Rat Brain**

Kainic acid (KA) is a potent agonist for the AMPA receptor. Peripheral injections of KA result in recurrent seizures and the subsequent degeneration of select populations of neurons in the hippocampus (Nadler et al., 1980; Schauwecker and Steward, 1997; Sperk et al., 1983). It has been shown that the activation of caspase-3 is a necessary component of KA-induced cell death (Faherty et al., 1999). To explore whether test compounds can block the neurotoxicity initiated by KA, the compounds can be subcutaneously injected (for example, at a concentration of 2 mg/kg) into C57BL/6 mice, followed by 25 mg/kg KA. In 5 days, the mice can be perfused and the brains cut to a thickness of 5 μm and mounted on slides. In the absence of a neuroprotective compound, TUNEL staining reveals that KA provokes enormous apoptosis in the hippocampus. If this apoptosis is substantially diminished by a test compound, this assay can demonstrate that the test compound is neuroprotective.

To further determine the neuroprotective potential *in vivo*, test compounds can be tested in a transient middle cerebral artery occlusion (MCAO) stroke model in adult male rats. After 2 h MCAO followed by reperfusion, the animals receive vehicle or test compounds (2 mg/kg) 5 min prior to the onset of reperfusion. If all or a substantial number of test animals survive the ischemic insult and treatment with the test compound, this will demonstrate that the compound is neuroprotective.

A representative brain slice stained with TTC 24 h after MCAO in vehicle-treated and compound-treated rats can be subjected to area and volume measurements from TTC sections to indicate whether treatments with the compound substantially reduces infarct volumes in this transient ischemic model of stroke. Positive results can be compared with those of NGF, which is known to reduce infarct volume and apoptosis in focal ischemia (Guegan et al., 1998).

LDF (laser-Doppler flowmetry) can be measured over the ipsilateral parietal cortex. One can measure the reduction of relative CBF (cerebral blood flow) within 5 minutes of MCAO in rats that subsequently received test or vehicle treatment.

Taken together, the assays described above can identify potent TrkA agonists, which are capable of preventing neuronal cell death and protecting the neurodegeneration elicited by excitatory neurotoxicity and stroke.

## II. Methods of Providing Neuroprotection Using TrkA Agonists

The selective TrkA agonists can be used to treat or prevent neurodegenerative disorders. The method involves administering to an animal an effective amount of a selective TrkA agonist, or a pharmaceutically acceptable salt or prodrug of the selective TrkA agonist, to provide neuroprotection, and to treat or prevent neurodegenerative diseases.

Selective TrkA agonists and partial agonists, such as those identified using the screening process described above, can be used to provide neuroprotection, before, during, or after a neural insult, such as a stroke or other ischemic event. The compounds neuroprotective effects can prevent loss of neuronal cell viability induced by exitotoxic agents in regions involved in memory encoding and exhibiting early degeneration in Alzheimer's disease and ischemia.

The selective TrkA agonists and partial agonists can also be used to treat demyelination disorders such as MS. While not wishing to be bound to a particular theory, it is believed that the selective TrkA agonists treat or prevent autoimmune demyelination, such as that observed in multiple sclerosis (MS), by inducing myelin protein genes.

The selective TrkA agonists and partial agonists can further be used to treat or prevent neurodegenerative disorders such as epilepsy, head and spinal chord trauma, Parkinson disease, Huntington's disease, Alzheimer's disease, or amyotrophic lateral schlerosis, or a neurological disorder. Additional neurological diseases that can be treated with the selective TrkA agonists are movement disorders, pain and the like.

The selective TrkA agonists or partial agonists can further be used to promote nerve cell survival, and can protect neural cells against cell death, for example, cell death due to the effects of neurotoxic agents.

In each of these methods, the TrkA agonist or partial agonists, or pharmaceutically acceptable salt or prodrug thereof, are administered to an animal an effective amount, optionally in a pharmaceutical composition, to provide neuroprotection, to treat demyelinating diseases, to enhance nerve regeneration, and/or to treat or prevent neurodegenerative diseases.

The compounds offer advantages over NGF, in that they are small molecules, and can be administered orally and prepared relatively inexpensively and in relatively

high purity, relative to NGF. Further, the compounds are more selective than NGF, by binding selectively to TrkA, rather than non-selectively to TrkA, TrkB, and TrkC.

## III. Representative Compounds

Gambogic Acid and Analogues Thereof

Gambogic acid and analogues thereof are known to have certain effects on apoptosis in cancer cells, and are believed to modulate (i.e., to agonize, partially agonize, or antagonize) the TrkA receptor, depending on the particular compound structure. The assays described herein can be used in a high-throughput manner to identify which compounds within this family of compounds have desired activity. Indeed, compounds with pronounced apoptotic activity may have considerable efficacy as anti-cancer drugs, whereas compounds with enhanced protective activity may have considerable efficacy for providing neuroprotection, treating neurodegenerative disorders, and the like.

The gambogic acid family (i.e., gambogic acid and analogues thereof) is described in more detail below.

Gambogic acid (GA) is the major active ingredient of gamboge, a brownish to orange resin exuded from Garcinia hanburryi tree in Southeast Asia. Pharmacokinetic study reveals that after intravenous injection, the $t_{1/2}$ of gambogic acid in human plasma is about 15.54 ± 1.3 h (Ding et al., 2007).

There are many functional groups in the structure of gambogic acid which can be modified. These include, but are not limited to, the carboxyl group, which can be converted to an ester, amide, ketone or alcohol and other functional groups; the ester and amide, in turn, may also contain other functional groups, such as the carboxyl of an amino acid, which can be further modified; the hydroxy group, may be converted to an ether, ester or other functional groups; the carbon-carbon double bond between C-9 and C-10 is part of an α., β-unsaturated ketone, which can react with a nucleophile, be reduced to a carbon-carbon single bond, or may be converted to an epoxide, which in turn may undergo further reaction; the carbon-carbon double bond between C-27 and C-28 is part of an α., β-unsaturated carboxyl, that may also react with a nucleophile, be reduced to a carbon-carbon single bond, or may be converted to a cyclopropane ring, which in turn may undergo further reaction; the two isoprene carbon-carbon double bonds at C-37/C-38 and C-32/C-33, may also be reduced to a

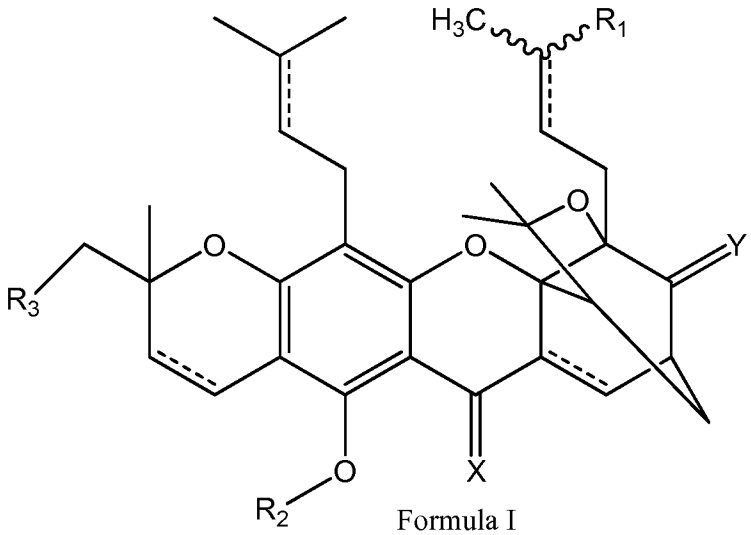
carbon-carbon single bond, be cleaved to form an aldehyde group or a carboxyl group, both of which may be modified to other functional groups, or be converted to an epoxide, which in turn may undergo further reaction; the carbon-carbon double bond between C-3 and C-4 may also be reduced to a carbon-carbon single bond, or be converted to an epoxide that may undergo further reaction; the ketone group at C-12 may be reduced to an alcohol, or may be converted to an oxime, a semicarbazone, or an amino group; the other ketone group may also be reduced, or may be converted to other functional groups. In short, many derivatives of gambogic acid can be prepared.

In addition, analogs of gambogic acid, including isomorellin, morellic acid, desoxymorellin, gambogin, morelline dimethyl acetal, isomoreollin B Moreollic acid, gambogenic acid, gambogenin, isogambogenin, desoxygambogenin, gambogenin dimethyl acetal, gambogellic acid, hanburin (Asano, J., et al., Phytochemistry 41:815-820 (1996)), isogambogic acid, isomorellinol (Lin, L. -J., et al., Magn. Reson. Chem. 31:340-347 (1993)) and neo-gambogic acid (Lu, G. B., et al., Yao Hsueh Hsueh Pao 19:636-639 (1984)) can be isolated from gamboge. Other analogs of gambogic acid, including morellin, desoxymorellin, dihydroisomorellin (Bhat et al. Indian J. Chem. 2:405-409 (1964)) and moreollin (Rao et al. Proc. Indian Acad. Sci. 87A:75-86 (1978)), can be isolated from the seed of Garcinia morella. Morellinol can be isolated from the bark of Garcinia morella (Adawadkar et al. Indian J. Chem. 14B:19-21 (1976)). Gaudichaudiones (A-H) and gaudichaudiic acids A-E can be isolated from the leaves of Garcinia gaudichaudii (Guttiferae) (Cao, S. -G., et al., Tetrahedron 54(36):10915-10924 (1998) and Cao, S. -G., et al., Tetrahedron Lett. 39(20):3353-3356 (1998)), and forbesione can be isolated from Garcinia forbesii (Leong, Y. -W., et al., J. Chem. Res., Synop. 392-393 (1996)).

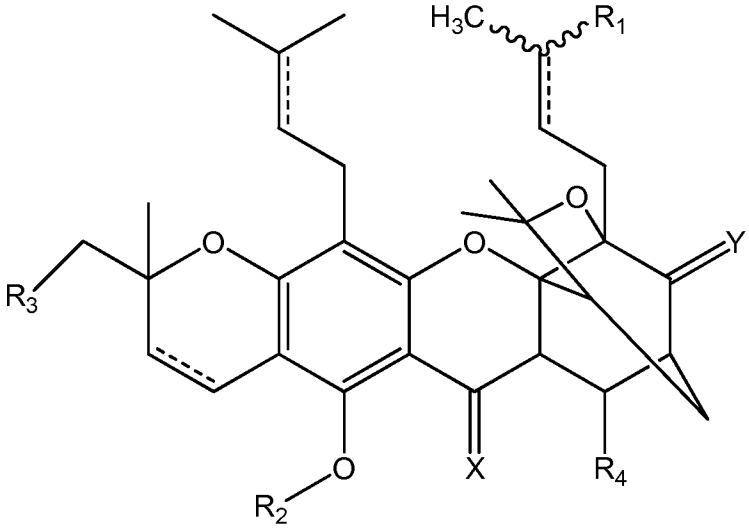
Bractatin, isobractatin, 1-0-methylbractatin, 1-0-methylisobractatin, 1-0-methyl-8-methoxy-8,8a-dihydrobractatin, and 1-0-methylneobractatin can be isolated from a leaf extract of G. bracteata (Thoison, O., et al., J. Nat. Prod. 63:441 446 (2000)). Novel gaudichaudiic acids (F-I) can be isolated from the bark of Indonesian Garcinia gaudichaudil (Xu, Y., et al., Organic Lett. 2(24):3945 3948 (2000)). Scortechinones (A-C) can be isolated from twigs of Garcinia scortechinii (Rukachaisirikul, V., et al., Tetrahedron 56:8539 8543 (2000)). Gaudispirolactone can be isolated from the bark of Garcinia gaudichaudii (Wu, J., et al., Tetrahedron Lett.

42:727 729 (2001)). These gambogic acid analogs also can be used to prepare derivatives similar to gambogic acid.

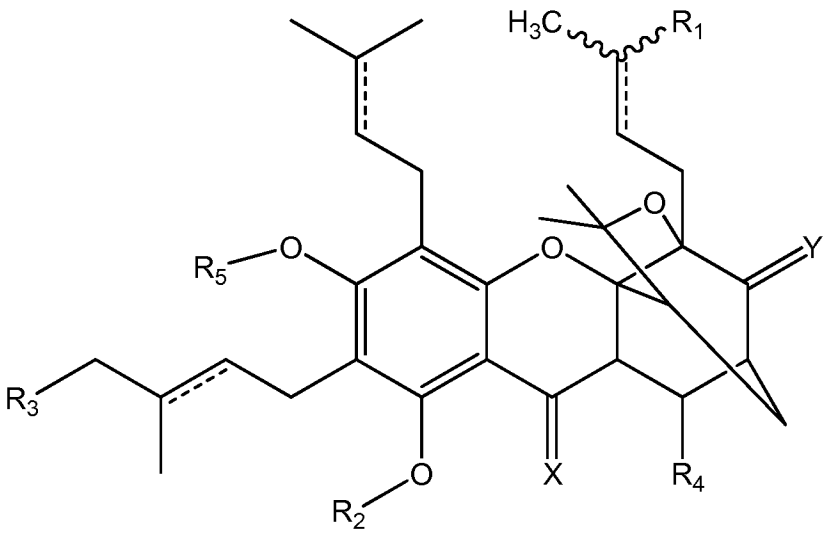
Specifically, compounds useful in this aspect of the present invention are gambogic acid, its analogs and derivatives as represented by Formulae I-III:




Formula I




Formula II




Formula III

or pharmaceutically acceptable salts or prodrugs thereof, wherein: the dotted lines are single bonds, double bonds or an epoxy group;

X together with the attached carbon is a methylene, carbonyl, hydroxymethinyl, alkoxymethinyl, aminomethinyl, an oxime, a hydrazone, an arylhydrazone or semicarbazone;

Y together with the attached carbon is a methylene, carbonyl, hydroxymethinyl, alkoxymethinyl, aminomethinyl, an oxime, a hydrazone, an arylhydrazone or semicarbazone;

$R_1$ is formyl, methylenehydroxy, carboxy, acyl ($R_a$ CO), optionally substituted alkoxycarbonyl ($R_a$ OCO), optionally substituted alkylthiocarbonyl, optionally substituted aminocarbonyl (carbamyl, $R_b$ $R_c$ NCO) or hydroxyaminocarbonyl,

where $R_a$ is hydrogen, optionally substituted lower alkyl, optionally substituted aryl, optionally substituted lower aralkyl group, or N-succinimidyl; or $R_a$ is the group $-(CH_2CH_2O)_nR_m$ wherein n=1 10 and $R_m$ is hydrogen or $C_{1-10}$ alkyl;

$R_b$ and $R_c$ are independently hydrogen, optionally substituted heteroalkyl, optionally substituted lower alkyl, optionally substituted aryl, optionally substituted heteroaryl or optionally substituted lower aralkyl groups; or $R_b$ and $R_c$ may be taken together with the attached N to form an optionally substituted, saturated or partially saturated 5-7 membered heterocyclo group, including piperidine, morpholine and piperazine; or $R_a$ is the group $--(CH_2CH_2O)_{nR.m}$ wherein n=1 10 and $R._m$ is hydrogen or $C_{1-10}$ alkyl; or $R_b$ and $R_c$ may be taken together with the attached N to form a heterocycle, including piperidine, morpholine and piperazine;

$R_2$ is hydrogen, optionally substituted alkyl, acyl ($R_a$ CO), carbamyl ($R_b$ $R_c$ NCO) or sulfonyl ($R_d$ $SO_2$), where $R_a$, $R_b$ and $R_c$ are defined above;

$R_d$ is hydrogen, optionally substituted lower alkyl, optionally substituted aryl, optionally substituted lower aralkyl groups; ($R_bR_cNCO$), halogen, hydroxy, optionally substituted alkyl, cycloalkyl, alkoxy, arylalkoxy, aryloxy, heteroaryloxy, alkylthio, arylalkylthio, arylthio, heteroarylthio, amino, aminoalkoxy, optionally substituted

saturated or partially saturated heterocyclo, heterocycloalkoxy or heterocycloalkylamino;

$R_3$ is hydrogen or prenyl; and

$R_5$ is hydrogen, optionally substituted alkyl or acyl ($R_a$ CO), carbamyl ($R_b$ $R_c$ NCO) or sulfonyl ($R_d$ $SO_2$), where $R_a$, $R_b$, $R_c$ and $R_d$ are defined above.

Exemplary compounds falling within the scope of Formula I include compounds wherein $R_1$ is formyl, acetyl, propionyl, carboxy, methoxycarbonyl, ethoxycarbonyl, methylthiocarbonyl, ethylthiocarbonyl, butylthiocarbonyl, dimethylcarbamyl, diethylcarbamoyl, 1-piperidinylcarbonyl, N-methyl-N'-piperazinylcarbonyl, 2-(dimethylamino)ethylcarbamyl or N-morpholinylcarbonyl; $R_2$ is hydrogen, formyl, acetyl, dimethylcarbamyl, diethylcarbamyl, 2-(dimethylamino)ethylcarbamyl, 1-piperidinylcarbonyl, N-methyl-N'-piperazinylcarbonyl, N-morpholinyl-carbonyl, methylsulfonyl, ethylsulfonyl, phenylsulfonyl, methyl, ethyl, 2-piperidinylethyl, 2-morpholinylethyl, 2-(dimethylamino)ethyl, or 2-(diethylamino)ethyl; X and Y is O; $R_3$ is prenyl; and the dotted lines are double bonds or an epoxy group. If the double bond is present at C27-28, it is preferred that it has the Z configuration.

Exemplary compounds falling within the scope of Formula II include compounds wherein $R_1$ is formyl, acetyl, propionyl, carboxy, methoxycarbonyl, ethoxycarbonyl, methylthiocarbonyl, ethylthiocarbonyl, butylthiocarbonyl, dimethylcarbamyl, diethylcarbamyl, N-piperidinylcarbonyl, N-methyl-N'-piperazinylcarbonyl, 2-(dimethylamino)ethylcarboxy or N-morpholinylcarbonyl; $R_2$ is hydrogen, formyl, acetyl, dimethylcarbamyl, diethylcarbamyl, 2-(dimethylamino)ethylcarbamyl, 1-piperidinylcarbonyl, N-methyl-N'-piperazinylcarbonyl, N-morpholinylcarbonyl, methylsulfonyl, ethylsulfonyl, phenylsulfonyl, methyl, ethyl, 2-piperidinylethyl, 2-morpholinylethyl, 2-(dimethylamino)ethyl, or 2-(diethylamino)ethyl; and $R_d$ is methyl, ethyl, phenyl, chloro, bromo, hydroxy, hydrogen, methoxy, ethoxy, methylthio, ethylthio, butylthio, dimethylamino, diethylamino, piperidinyl, pyrrolidinyl, imidazolyl, pyrazolyl, N-methylpiperazinyl, 2-(dimethylamino)ethylamino or morpholinyl; $R_d$ is chloro, bromo, hydroxy, hydrogen, methoxy, ethoxy, phenoxy, benzyloxy, methylthio, ethylthio, butylthio, phenylthio, dimethylamino, diethylamino, piperidinyl,

piperazinyl, pyrrolidinyl, imidazolyl, pyrazolyl, N-methylpiperazinyl, 2-(dimethylamino)ethylamino, morpholinyl, anilino, 4-acetylpiperazinyl, 2-(morpholinyl)-ethylamino, 4-(2-pyridyl)piperazinyl, 2-(morpholinyl)ethoxy, or 2-dimethyl-aminoethoxy; X and Y is O; $R_3$ is prenyl; and the dotted lines are double bonds. If the double bond is present at C27-28, it is preferred that it has the Z configuration.

Exemplary compounds falling within the scope of Formula III include compounds wherein $R_1$ is formyl, acetyl, propionyl, carboxy, methoxycarbonyl, ethoxycarbonyl, methylthiocarbonyl, ethylthiocarbonyl, butylthiocarbonyl, dimethylcarbamyl, diethylcarbamyl, N-piperidinylcarbonyl, N-methyl-N'-piperazinylcarbonyl, 2-(dimethylamino)ethylcarbamyl or N-morpholinylcarbonyl; $R_2$ is hydrogen, formyl, acetyl, dimethylcarbamyl, diethylcarbamyl, 2-(dimethylamino)ethylcarbamyl, 1-piperidinylcarbonyl, N-methyl-N'-piperazinylcarbonyl, N-morpholinylcarbonyl, methylsulfonyl, ethylsulfonyl, phenylsulfonyl, methyl, ethyl, 2-piperidinylethyl, 2-morpholinylethyl, 2-(dimethylamino)ethyl, or 2-(diethylamino)ethyl; $R_5$ is hydrogen, formyl, acetyl, dimethylcarbamyl, diethylcarbamyl, 2-(dimethylamino)ethylcarbamyl, 1-piperidinylcarbonyl, N-methyl-N'-piperazinylcarbonyl, N-morpholinylcarbonyl, methylsulfonyl, ethylsulfonyl, phenylsulfonyl, methyl, ethyl, 2-piperidinylethyl, 2-morpholinylethyl, 2-(dimethylamino)ethyl, or 2-(diethylamino)ethyl; X and Y is O; $R_3$ is prenyl; and the dotted lines are double bonds. If the double bond is present at C27-28, it is preferred that it has the Z configuration.

Exemplary compounds that can be screened using the screening methods described herein include, without limitation: Gambogic acid; Methyl gambogate; 9,10-Dihydrogambogic acid; 9,10-Dihydrogambogyl (4-methylpiperazine); 9,10-Dihydrogambogyl (2-dimethylaminoethylamine); Gambogyl diethylamine; Gambogyl dimethylamine; Gambogyl amine; Gambogyl hydroxyamine; Gambogyl piperidine; 6-Methoxy-gambogic acid; 6-(2-Dimethylaminoethoxy)-gambogic acid; 6-(2-Piperidinylethoxy)-gambogic acid; 6-(2-Morpholinylethoxy)-gambogic acid; 6-Methoxy-gambogyl piperidine; Gambogyl morpholine; Gambogyl (2-dimethylaminoethylamine); 10-Morpholinyl-gambogyl morpholine; 10-Morpholinyl-gambogyl piperidine; 10-(4-Methylpiperazinyl)-gambogyl piperidine; 10-(4-

Methylpiperazinyl)-gambogyl morpholine; 10-Piperidinyl-gambogyl piperidine; 10-(4-Methylpiperazinyl)-gambogyl (4-methylpiperazine); Gambogyl (4-methylpiperazine); Methyl-6-methoxy-gambogate; Gambogenic acid; Gambogenin; 10-Methoxy-gambogic acid; 10-Butylthio-gambogic acid; 10-(4-Methylpiperazinyl)-gambogic acid; 10-Pyrrolidinyl-gambogic acid; Methyl-10-Morpholinyl-gambogate; 10-Piperidinyl-gambogic acid; 10-Morpholinyl-gambogic acid; N-(2-Gambogylamidoethyl)biotinamide; Gambogyl (2-morpholinylethylamine); 9,10-Epoxygambogic acid; Gambogyl (4-(2-pyridyl)piperazine); 10-(4-(2-Pyridyl)piperazinyl)gambogyl (4-(2-pyridyl)piperazine); 6-Acetylgambogic acid; 10-(4-(2-Pyridyl)piperazinyl)gambogic acid; N-Hydroxysuccinimidyl gambogate; 8-(Gambogylamido)octanoic acid; 6-(Gambogylamido)hexanoic acid; 12-(Gambogylamido)dodecanoic acid; N-Hydroxysuccinimidyl-8-(gambogylamido)octanoate; N-Hydroxysuccinimidyl-6-(gambogylamido)hexanoate; N-Hydroxysuccinimidyl-12-(gambogylamido)dodecanoate; 10-Methoxy-gambogyl piperidine; Gambogyl (4-(2-pyrimidyl)piperazine); Gambogyl (bis(2-pyridylmethyl)amine); Gambogyl (N-(3-pyridyl)-N-(2-hydroxybenzyl)amine); Gambogyl (4-benzylpiperazine); Gambogyl (4-(3,4-methylenedioxybenzyl)piperazine); Gambogyl (N-methyl-5-(methylamino)-3-oxapentylamine); Gambogyl (N-methyl-8-(methylamino)-3,6-dioxaoctylamine); Gambogyl (N-ethyl-2-(ethylamino)ethylamine); Gambogyl (4-isopropylpiperazine); Gambogyl (4-cyclopentylpiperazine); Gambogyl (N-(2-oxo-2-ethoxyethyl)-(2-pyridyl)methylamine); Gambogyl (2,5-dimethylpiperazine); Gambogyl (3,5-dimethylpiperazine); Gambogyl (4-(4-acetylphenyl)piperazine); Gambogyl (4-ethoxycarbonylpiperazine); Gambogyl (4-(2-oxo-2-pyrrolidylethyl)piperazine); Gambogyl (4-(2-hydroxyethyl)piperazine); Gambogyl (N-methyl-2-(methylamino)ethylamine); Gambogyl (N-methyl-2-(benzylamino)ethylamine); Gambogyl (N-methyl-(6-methyl-2-pyridyl)methylamine); Gambogyl (N-ethyl-2-(2-pyridyl)ethylamine); Gambogyl (N-methyl-(2-pyridyl)methylamine); Gambogyl (N-methyl-4-(3-pyridyl)butylamine); Gambogyl (bis(3-pyridylmethyl)amine); Gambogyl (2,4-dimethyl-2-imidazoline); Gambogyl (4-methyl-homopiperazine); Gambogyl (4-(5-hydroxy-3-oxapentyl)piperazine); Gambogyl (3-dimethylaminopyrrolidine); Gambogyl ((2-furanyl)methylamine); Gambogyl (2-hydroxy-1-methyl-2-phenylethylamine); Gambogyl (3,4,5-trimethoxybenzylamine); Gambogyl (2-(2-methoxyphenyl)ethylamine); Gambogyl (2-methoxybenzylamine); Gambogyl (3,4-

methylenedioxybenzylamine); Gambogyl (2-(2,5-dimethoxyphenyl)ethylamine); Gambogyl (2-(3-methoxyphenyl)ethylamine); Gambogyl (3-(piperidinyl)propylamine); Gambogyl (2-(piperidinyl)ethylamine); Gambogyl (3,4-dimethoxybenzylamine); Gambogyl ((2-tetrahydrofuranyl)methylamine); Gambogyl ((N-ethyl-2-pyrrolidinyl)methylamine); Gambogyl (2-diethylaminoethylamine); Gambogyl (2,2-dimethyl-3-dimethylaminopropylamine); Gambogyl ((N-ethoxycarbonyl-4-piperidinyl)amine); Gambogyl (2-carbamylpyrrolidine); Gambogyl (3-(homopiperidinyl)propylamine); Gambogyl ((N-benzyl-4-piperidinyl)amine); Gambogyl (2-(4-methoxyphenyl)ethylamine); Gambogyl (4-oxa-hex-5-enylamine); Gambogyl (6-hydroxyhexylamine); Gambogyl (2-(3,5-dimethoxyphenyl)ethylamine); Gambogyl (3,5-dimethoxybenzylamine); and Gambogyl (2-carbamyl-2-(4-hydroxyphenyl)ethylamine).

9,10-Dihydro-10-morpholinyl-gambogyl (N-methylpiperazine); 9,10-Dihydro-10-piperidinyl-gambogyl (N-methylpiperazine); 9,10-Dihydro-10-[2-(morpholinyl)ethylamino]-gambogyl (N-methylpiperazine); 9,10-Dihydro-10-[4-(2-pyridyl)piperazinyl]-gambogyl (N-methylpiperazine); 9,10-Dihydro-10-[2-(morpholinyl)ethoxy]-gambogyl (N-methylpiperazine); 9,10-Dihydro-10-(2-dimethylaminoethoxy)-gambogyl (N-methylpiperazine); 9,10-Dihydro-10-morpholinyl-gambogyl morpholine; 9,10-Dihydro-10-ethoxy-gambogyl piperidine; 9,10-Dihydro-10-morpholinyl-gambogyl (dimethylamine); Ethyl 9,10-dihydro-10-morpholinyl-gambogate; Methyl 9,10-dihydro-10-benzyloxy-gambogate; Methyl 9,10-dihydro-10-(4-acetylpiperazinyl)-gambogate; Methyl 9,10-dihydro-10-(piperidinyl)-gambogate; 9,10-Dihydro-10-[4-(2-pyridyl)piperazinyl]-gambogyl (diethylamine); 9,10-Dihydro-10-[4-(2-pyridyl)piperazinyl]-gambogyl (methylamine); 9,10-Dihydro-10-(morpholinyl)-gambogyl (diethylamine); Methyl-9,10-dihydro-10-ethoxy-gambogate; 9,10-Dihydro-10-ethoxy-gambogic acid; 9,10-Dihydro-10-ethoxy-gambogyl (diethylamine); Ethyl 9,10-dihydro-10-ethoxy-gambogate; Methyl 9,10-dihydro-10-(4-methylpiperazinyl)-gambogate; Ethyl 9,10-dihydro-10-(piperidinyl)-gambogate; Ethyl 9,10-dihydro-10-(4-methylpiperazinyl)-gambogate; and Ethyl 9,10-dihydro-10-(4-acetylpiperazinyl)-gambogate.

The positions in gambogic acid are numbered according to Asano, J., et al., Phytochemistry 41:815 820 (1996), and Lin, L.-J., et al., Magn. Reson. Chem. 31:340 347 (1993).

Within this list of compounds, exemplary compounds may include:

9,10-Dihydro-10-morpholinyl-gambogyl morpholine; 9,10-Dihydro-10-morpholinyl-gambogyl piperidine; 9,10-Dihydro-10-(4-methylpiperazinyl)-gambogyl piperidine; 9,10-Dihydro-10-(4-methylpiperazinyl)-gambogyl morpholine; 9,10-Dihydro-10-piperidinyl-gambogyl piperidine; 9,10-Dihydro-10-(4-methylpiperazinyl)-gambogyl methylpiperazine); 9,10-Dihydro-10-methoxy-gambogic acid; 9,10-Dihydro-10-butylthio-gambogic acid; 9,10-Dihydro-10-(4-methylpiperazinyl)-gambogic acid; 9,10-Dihydro-10-pyrrolidinyl-gambogic acid; Methyl 9,10-Dihydro-10-morpholinyl-gambogate; 9,10-Dihydro-10-piperidinyl-gambogic acid; 9,10-Dihydro-10-morpholinyl-gambogic acid; 9,10-Dihydro-10-(4-(2-pyridyl)piperazinyl)gambogyl (4-(2-pyridyl)piperazine); 9,10-Dihydro-10-(4-(2-pyridyl)piperazinyl)gambogic acid; and 9,10-Dihydro-10-methoxy-gambogyl piperidine.

Within this list of compounds, exemplary compounds may also include:

2-{2-[2-(2-Octyloxyethoxy)ethoxy]ethoxy}ethyl Gambogate; 2-{2-[2-(2-Octyloxyethoxy)ethoxy]ethoxy}ethyl 9,10-Dihydro-10-morpholinyl gambogate; 2-[2-(2-Ethoxyethoxy)ethoxy]ethyl Gambogate; 2-[2-(2-Ethoxyethoxy)ethoxy]ethyl 9,10-Dihydro-10-morpholinyl gambogate; Propyl Gambogate; Propyl 9,10-Dihydro-10-morpholinyl-gambogate; 2-{2-[2-(2-Methoxyethoxy)ethoxy]ethoxy}ethyl gambogate; 2-{2-[2-(2-Methoxyethoxy)ethoxy]ethoxy}ethyl 9,10-Dihydro-10-morpholinyl-gambogate; 2-Hydroxyethyl Gambogate; 2-Hydroxyethyl 9,10-Dihydro-10-morpholinyl-gambogate; Methyl 9,10-Dihydro-gambogate; Methyl 9,10,12-Trihydro-12-hydroxy-gambogate; Methyl 32,33-Epoxy-37,38-epoxy-gambogate; Methyl 37,38-Epoxy gambogate; Methyl 9,10-Epoxy-gambogate; Butyl Gambogate; Isobutyl Gambogate; Butyl 9,10-Dihydro-10-morpholinyl-gambogate; Isobutyl 9,10-Dihydro-10-morpholinyl-gambogate; 3,4,9,10,32,33,37,38-Octahydro-gambogic Acid; Ethyl 3,4,9,10,32,33,37,38-Octahydro-10-morpholinyl-gambogate; Ethyl 3,4,32,33,37,38-Hexahydro-gambogate; Ethyl 12-Hydro-12-hydroxy-gambogate; Ethyl 9,10,12-Trihydro-12-hydroxy-gambogate; Ethyl 3,4,9,10,27,28,32,33,37,38-Decahydro-10-morpholinyl-gambogate; and Ethyl 3,4,27,28,32,33,37,38-Octahydro-gambogate.

Still other exemplary compounds may include:

9,10-Dihydro-10-morpholinyl-gambogyl (N-methylpiperazine); 9,10-Dihydro-10-piperidinyl-gambogyl (N-methylpiperazine); 9,10-Dihydro-10-[2-(morpholinyl)ethylamino]-gambogyl (N-methylpiperazine); 9,10-Dihydro-10-[4-(2-pyridyl)piperazinyl]-gambogyl (N-methylpiperazine); 9,10-Dihydro-10-[2-(morpholinyl)ethoxy]-gambogyl (N-methylpiperazine); 9,10-Dihydro-10-(2-dimethylaminoethoxy)-gambogyl (N-methylpiperazine); 9,10-Dihydro-10-morpholinyl-gambogyl morpholine; 9,10-Dihydro-10-ethoxy-gambogyl piperidine; 9,10-Dihydro-10-morpholinyl-gambogyl (dimethylamine); Ethyl 9,10-dihydro-10-morpholinyl-gambogate; Methyl 9,10-dihydro-10-benzyloxy-gambogate; Methyl 9,10-dihydro-10-(4-acetylpiperazinyl)-gambogate; Methyl 9,10-dihydro-10-(piperidinyl)-gambogate; 9,10-Dihydro-10-[4-(2-pyridyl)piperazinyl]-gambogyl (diethylamine); 9,10-Dihydro-10-[4-(2-pyridyl)piperazinyl]-gambogyl (methylamine); 9,10-Dihydro-10-(morpholinyl)-gambogyl (diethylamine); Methyl-9,10-dihydro-10-ethoxy-gambogate; 9,10-Dihydro-10-ethoxy-gambogic acid; 9,10-Dihydro-10-ethoxy-gambogyl (diethylamine); Ethyl 9,10-dihydro-10-ethoxy-gambogate; Methyl 9,10-dihydro-10-(4-methylpiperazinyl)-gambogate; Ethyl 9,10-dihydro-10-(piperidinyl)-gambogate; Ethyl 9,10-dihydro-10-(4-methylpiperazinyl)-gambogate; and Ethyl 9,10-dihydro-10-(4-acetylpiperazinyl)-gambogate.

Other exemplary compounds include:

2-{2-[2-(2-Octyloxyethoxy)ethoxy]ethoxy}ethyl 9,10-Dihydro-10-morpholinyl gambogate; 2-[2-(2-Ethoxyethoxy)ethoxy]ethyl 9,10-Dihydro-10-morpholinyl gambogate; Propyl 9,10-Dihydro-10-morpholinyl-gambogate; 2-{2-[2-(2-Methoxyethoxy)ethoxy]ethoxy}ethyl 9,10-Dihydro-10-morpholinyl-gambogate; 2-Hydroxyethyl 9,10-Dihydro-10-morpholinyl-gambogate; Methyl 9,10-Epoxy-gambogate; Butyl 9,10-Dihydro-10-morpholinyl-gambogate; Isobutyl 9,10-Dihydro-10-morpholinyl-gambogate; Ethyl 3,4,9,10,32,33,37,38-Octahydro-10-morpholinyl-gambogate; Ethyl 3,4,9,10,27,28,32,33,37,38-Decahydro-10-morpholinyl-gambogate; and 9,10-Dihydro-10-(morpholinyl)-gambogyl methylamine; Ethyl gambogate; and Gambogyl methylamine; 2-{2-[2-(2-Octyloxyethoxy)ethoxy]ethoxy}ethyl Gambogate; 2-[2-(2-Ethoxyethoxy)ethoxy]ethyl Gambogate; Propyl Gambogate; 2-

{2-[2-(2-Methoxyethoxy)ethoxy]ethoxy}ethyl gambogate; 2-Hydroxyethyl Gambogate; Methyl 9,10-Dihydro-gambogate; Methyl 9,10,12-Trihydro-12-hydroxy-gambogate; Methyl 32,33-Epoxy-37,38-epoxy-gambogate; Methyl 37,38-Epoxy gambogate; Butyl Gambogate; Isobutyl Gambogate; 3,4,9,10,32,33,37,38-Octahydro-gambogic Acid; Ethyl 3,4,32,33,37,38-Hexahydro-gambogate; Ethyl 12-Hydro-12-hydroxy-gambogate; Ethyl 9,10,12-Trihydro-12-hydroxy-gambogate; and Ethyl 3,4,27,28,32,33,37,38-Octahydro-gambogate.

Some compounds within this family include polyether substituents, such as substituted polyethyleneglycol, known as PEG. PEGs are water soluble polymers that impart unique physio-chemical properties to compounds and polymers, thus expanding the potential uses of the compounds and polymers. For example, PEG-modified proteins exhibit improved pharmacological performance over non-PEG-modified proteins. See, for example, Delgado, C. et al., Crit. Rev. Ther. Drug Carrier Syst. 9:249 304 (1992). PEG-modified liposomes also exhibit unique properties such as increased permiabilities. Sriwongsitanont, S. and Ueno, M., Chem. Pharm. Bull. 50:1238 1244 (2002). Modifying compounds of the present invention with PEG groups, increases their solubilty thus reducing their systemic toxicity and their toxicity at the site of administration. Particular PEGs for use in the present invention have the formula $--(CH_2CH_2O)_{,nR.m}$ wherein n=1 10 and $R._m$ is hydrogen or $C_1$-10 alkyl. Preferred PEGs include, but are not limited to, $HOCH_2CH_2OH$, $CH_3CH_2(OCH_2CH_2)_3OH$, $CH_3(OCH_2CH_2)_dOH$, and $CH_3(CH_2)_7(OCH_2CH_2)_dOH$.

Optional substituents on the alkyl groups include one or more halo, hydroxy, carboxyl, alkoxycarbonyl, amino, nitro, cyano, $C_1$ -$C_6$ acylamino, $C_1$ -$C_6$ aminoacyl, $C_1$ -$C_6$ acyloxy, $C_1$ -$C_6$ alkoxy, aryloxy, alkylthio, $C_6$ -$C_1$0 aryl, $C_d$ -$C_7$ cycloalkyl, $C_2$ -$C_6$ alkenyl, $C_2$ -$C_6$ alkynyl, $C_6$ -$C_1$0 aryl($C_2$ -$C_6$)alkenyl, $C_6$ -$C_1$0 aryl($C_2$ -$C_6$)alkynyl, saturated or partially saturated 5-7 membered heterocyclo group, or heteroaryl.

Useful alkyl groups include straight-chained and branched $C_1$-10 alkyl groups, more preferably $C_1$-6 alkyl groups. Typical $C_1$-10 alkyl groups include methyl, ethyl, propyl, isopropyl, butyl, sec-butyl, tert-butyl, 3-pentyl, hexyl and octyl groups, which may be optionally substituted.

Useful alkoxy groups include oxygen substituted by one of the $C_1$-10 alkyl groups mentioned above, which may be optionally substituted.

Useful alkylthio groups include sulphur substituted by one of the $C_1$-10 alkyl groups mentioned above, which may be optionally substituted. Also included are the sulfoxides and sulfones of such alkylthio groups.

Useful amino groups include --$NH_2$, --$NHR_11$, and --$NR_11$ $R_12$, wherein $R_11$ and $R_12$ are $C_1$-10 alkyl or cycloalkyl groups, or $R_11$ and $R_12$ are combined with the N to form a ring structure, such as a piperidine, or $R_11$ and $R_12$ are combined with the N and another heteroatom to form an optionally substituted, saturated or partially saturated 5-7 membered heterocyclo group, such as a piperazine. The alkyl group may be optionally substituted.

Useful heteroatoms include N, O or S.

Optional substituents on the aryl, aralkyl and heteroaryl groups include one or more acyl, alkylenedioxy (--$OCH_2$ O--), halo, $C_1$ -$C_6$ haloalkyl, $C_6$ -$C_10$ aryl, $C_d$ -$C_7$ cycloalkyl, $C_1$ -$C_6$ alkyl, $C_2$ -$C_6$ alkenyl, $C_2$ -$C_6$ alkynyl, $C_6$ -$C_10$ aryl($C_1$ -$C_6$)alkyl, $C_6$ -$C_10$ aryl($C_2$ -$C_6$)alkenyl, $C_6$ -$C_10$ aryl($C_2$ -$C_6$)alkynyl, $C_1$ -$C_6$ hydroxyalkyl, nitro, amino, ureido, cyano, $C_1$ -$C_6$ acylamino, hydroxy, thiol, $C_1$ -$C_6$ acyloxy, azido, $C_1$ -$C_6$ alkoxy, or carboxy.

Useful heteroalkyl groups contain 1-10 carbon atoms and 1, 2 or 3 heteroatoms. Examples of heteroalkyl groups include --$CH_2$ $CH_2$ O $CH_2$ $CH_3$, --$CH_2$ $CH_2$ $OCH_2$ $CH_2$ $OCH_2$ $CH_3$, --$CH_2$ $CH_2$ $NHCH_3$, --$CH_2$ $CH_2$ $N(CH_2$ $CH_3)_2$, --$CH_2$ $CH_2$ $OCH_2$ $CH_2$ $NHCH_3$, --$CH_2$ $CH_2$ $OCH_2$ $CH_2$ $OCH_2$ $CH_2$ $NHCH_3$, --$CH_2$ $CH_2$ $NHCH_2$ $CH_3$, --$CH_2$ $C(CH_3)_2$ $CH_2$ $N(CH_3)_2$ or --$CH_2$ (N-ethylpyrrolidine), which may be optionally substituted.

Optional substituents on heteroalkyl groups include one or more halo, hydroxy, carboxyl, amino, nitro, cyano, alkyl, $C_1$ -$C_6$ acylamino, $C_1$ -$C_6$ aminoacyl, $C_1$ -$C_6$ acyloxy, $C_1$ -$C_6$ alkoxy, aryloxy, alkylthio, $C_6$ -$C_10$ aryl, $C_d$ -$C_7$ cycloalkyl, $C_2$ -$C_6$ alkenyl, alkenoxy, $C_2$ -$C_6$ alkynyl, $C_6$ -$C_10$ aryl($C_2$ -$C_6$)alkenyl, $C_6$ -$C_10$ aryl($C_2$ -$C_6$)alkynyl, saturated and unsaturated heterocyclic, or heteroaryl.

Useful aryl groups are $C_6$-14 aryl, especially $C_6$-10 aryl. Typical $C_6$-14 aryl groups include phenyl, naphthyl, phenanthrenyl, anthracenyl, indenyl, azulenyl, biphenyl, biphenylenyl and fluorenyl groups.

Useful cycloalkyl groups are $C_3$-8 cycloalkyl. Typical cycloalkyl groups include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl and cycloheptyl.

Useful saturated or partially saturated carbocyclic groups are cycloalkyl groups as defined above, as well as cycloalkenyl groups, such as cyclopentenyl, cycloheptenyl and cyclooctenyl.

Useful halo or halogen groups include fluorine, chlorine, bromine and iodine.

Useful aralkyl groups include any of the above-mentioned $C_1$-10 alkyl groups substituted by any of the above-mentioned $C_6$-14 aryl groups. Useful values include benzyl, phenethyl and naphthylmethyl.

Useful haloalkyl groups include $C_1$-10 alkyl groups substituted by one or more fluorine, chlorine, bromine or iodine atoms, e.g. fluoromethyl, difluoromethyl, trifluoromethyl, pentafluoroethyl, 1,1-difluoroethyl, chloromethyl, chlorofluoromethyl and trichloromethyl groups.

Useful acylamino groups are any $C_1$-6 acyl (alkanoyl) attached to an amino nitrogen, e.g. acetamido, propionamido, butanoylamido, pentanoylamido, hexanoylamido as well as aryl-substituted $C_2$-6 substituted acyl groups.

Useful acyloxy groups are any $C_1$-6 acyl (alkanoyl) attached to an oxy (--O--) group, e.g. formyloxy, acetoxy, propionoyloxy, butanoyloxy, pentanoyloxy, hexanoyloxy and the like.

Useful saturated or partially saturated 5-7 membered heterocyclo groups include tetrahydrofuranyl, pyranyl, piperidinyl, piperazinyl, pyrrolidinyl, imidazolidinyl, imidazolinyl, indolinyl, isoindolinyl, quinuclidinyl, morpholinyl, isochromanyl, chromanyl, pyrazolidinyl pyrazolinyl, tetronoyl and tetramoyl groups.

Optional substitutents on the 5-7 membered heterocyclo groups include one or more heteroaryl, heterocyclo, alkyl, aralkyl, cycloalkyl, alkoxycarbonyl, carbamyl, aryl or $C_1$ -$C_6$ aminoacyl.

Useful heteroaryl groups include any one of the following: thienyl, benzo[b]thienyl, naphtho[2,3-b]thienyl, thianthrenyl, furanyl, pyranyl, isobenzofuranyl, chromenyl, xanthenyl, phenoxanthiinyl, 2H-pyrrolyl, pyrrolyl, imidazolyl, pyrazolyl, pyridyl, pyrazinyl, pyrimidinyl, pyridazinyl, indolizinyl, isoindolyl, 3H-indolyl, indolyl, indazolyl, purinyl, 4H-quinolizinyl, isoquinolyl, quinolyl, phthalzinyl, naphthyridinyl, quinozalinyl, cinnolinyl, pteridinyl, carbazolyl, $_b$eta.-carbolinyl, phenanthridinyl, acrindinyl, perimidinyl, phenanthrolinyl, phenazinyl, isothiazolyl, phenothiazinyl, isoxazolyl, furazanyl, phenoxazinyl, 1,4-dihydroquinoxaline-2,3-dione, 7-aminoisocoumarin, pyrido[1,2-a]pyrimidin-4-one, 1,2-benzoisoxazol-3-yl, benzimidazolyl, 2-oxindolyl and 2-oxobenzimidazolyl.

Where the heteroaryl group contains a nitrogen atom in a ring, such nitrogen atom may be in the form of an N-oxide, e.g. a pyridyl N-oxide, pyrazinyl N-oxide, pyrimidinyl N-oxide and the like.

Optional substituents on the heteroaryl groups include one or more heteroaryl, heterocyclo, alkyl, aralkyl, cycloalkyl, alkoxycarbonyl, carbamyl, aryl and $C_1$ -$C_6$ aminoacyl.

Certain compounds may exist as stereoisomers including optical isomers. The invention includes all stereoisomers and both the racemic mixtures of such stereoisomers as well as the individual enantiomers that may be separated according to methods that are well known to those of ordinary skill in the art.

Examples of pharmaceutically acceptable addition salts include inorganic and organic acid addition salts such as hydrochloride, hydrobromide, phosphate, sulphate, citrate, lactate, tartrate, maleate, fumarate, mandelate and oxalate; and inorganic and organic base addition salts with bases such as sodium hydroxy, Tris(hydroxymethyl)aminomethane (TRIS, tromethane) and N-methyl-glucamine.

Examples of prodrugs include the simple esters of carboxylic acid containing compounds (e.g. those obtained by condensation with a $C_1$-4 alcohol according to methods known in the art); esters of hydroxy containing compounds (e.g. those obtained by condensation with a $C_{1-4}$ carboxylic acid, $C_{3-6}$ dioic acid or anhydride thereof (e.g. succinic and fumaric anhydrides according to methods known in the art); imines of amino containing compounds (e.g. those obtained by condensation with a $C_1$-4 aldehyde or ketone according to methods known in the art); and acetals and ketals of alcohol containing compounds (e.g. those obtained by condensation with chloromethyl methyl ether or chloromethyl ethyl ether according to methods known in the art).

The compounds can be prepared and purified using methods known to those skilled in the art, or as described in U.S. Patent Nos.6,462,041; 6,613,762; and 7,176,234. Specifically, gambogic acid can be purified by 1) preparation of the pyridine salt of the crude extract from gamboge (resin from Garcinia hanburyi Hook) followed by repeated recrystallization of the salt in ethanol or 2) converting the salt to the free acid. Using this procedure, about 10% by weight of gambogic acid with purity >99% (HPLC) can be obtained from the crude extract.

**Gambogic Amides**

Gambogic amide (where the amide group is $CONH_2$) is the most active selective TrkA agonist identified in the initial screening of around 2000 gambogic acid analogues. Gambogic amide can be prepared by reacting gambogic acid, or the corresponding acid halide or anhydride, with ammonia under routine conditions for preparing amides from carboxylic acids.

Other amides (i.e., where the amide group is $CONHR^6$, where $R^6$ is hydrogen, optionally substituted lower alkyl, optionally substituted aryl, optionally substituted lower aralkyl groups, where the substituents are as described above for "substituted alkyl") can be prepared by reacting gambogic acid, or the corresponding acid halide or anhydride, with the corresponding amine. Such reactions are well known to those of skill in the art.

## IV. Pharmaceutical Compositions

The compounds described herein can be incorporated into pharmaceutical compositions and used to prevent a condition or disorder in a subject susceptible to such a condition or disorder, and/or to treat a subject suffering from the condition or disorder. The pharmaceutical compositions described herein include one or more of the honokiol analogues described herein, and/or pharmaceutically acceptable salts thereof. Optically active compounds can be employed as racemic mixtures, as pure enantiomers, or as compounds of varying enantiomeric purity.

The manner in which the compounds are administered can vary. The compositions are preferably administered orally (e.g., in liquid form within a solvent such as an aqueous or non-aqueous liquid, or within a solid carrier). Preferred compositions for oral administration include pills, tablets, capsules, caplets, syrups, and solutions, including hard gelatin capsules and time-release capsules. Compositions may be formulated in unit dose form, or in multiple or subunit doses. Preferred compositions are in liquid or semisolid form. Compositions including a liquid pharmaceutically inert carrier such as water or other pharmaceutically compatible liquids or semisolids may be used. The use of such liquids and semisolids is well known to those of skill in the art.

The compositions can also be administered via injection, i.e., intraveneously, intramuscularly, subcutaneously, intraperitoneally, intraarterially, intrathecally; and intracerebroventricularly. Intravenous administration is a preferred method of injection. Suitable carriers for injection are well known to those of skill in the art, and

include 5% dextrose solutions, saline, and phosphate buffered saline. The compounds can also be administered as an infusion or injection (e.g., as a suspension or as an emulsion in a pharmaceutically acceptable liquid or mixture of liquids).

The formulations may also be administered using other means, for example, rectal administration. Formulations useful for rectal administration, such as suppositories, are well known to those of skill in the art. The compounds can also be administered by inhalation (e.g., in the form of an aerosol either nasally or using delivery articles of the type set forth in U.S. Patent No. 4,922,901 to Brooks et al., the disclosure of which is incorporated herein in its entirety); topically (e.g., in lotion form); or transdermally (e.g., using a transdermal patch, using technology that is commercially available from Novartis and Alza Corporation). Although it is possible to administer the compounds in the form of a bulk active chemical, it is preferred to present each compound in the form of a pharmaceutical composition or formulation for efficient and effective administration.

The compounds can be incorporated into drug delivery devices such as nanoparticles, microparticles, microcapsules, and the like. Representative microparticles/nanoparticles include those prepared with cyclodextrins, such as pegylated cyclodextrins, liposomes, including small unilamellar vesicles, and liposomes of a size designed to lodge in capillary beds around growing tumors. Suitable drug delivery devices are described, for example, in Heidel JD, et al., Administration in non-human primates of escalating intravenous doses of targeted nanoparticles containing ribonucleotide reductase subunit M2 siRNA, Proc Natl Acad Sci U S A. 2007 Apr 3;104(14):5715-21; Wongmekiat et al., Preparation of drug nanoparticles by co-grinding with cyclodextrin: formation mechanism and factors affecting nanoparticle formation, Chem Pharm Bull (Tokyo). 2007 Mar;55(3):359-63; Bartlett and Davis, Physicochemical and biological characterization of targeted, nucleic acid-containing nanoparticles, Bioconjug Chem. 2007 Mar-Apr;18(2):456-68;; Villalonga et al., Amperometric biosensor for xanthine with supramolecular architecture, Chem Commun (Camb). 2007 Mar 7;(9):942-4; Defaye et al., Pharmaceutical use of cyclodextrines: perspectives for drug targeting and control of membrane interactions, Ann Pharm Fr. 2007 Jan;65(1):33-49; Wang et al., Synthesis of Oligo(ethylenediamino)-beta-Cyclodextrin Modified Gold Nanoparticle as a DNA Concentrator; Mol Pharm. 2007 Mar-Apr;4(2):189-98; Xia et al., Controlled synthesis of Y-junction polyaniline nanorods and nanotubes using in situ self-assembly of

magnetic nanoparticles, J Nanosci Nanotechnol., 2006 Dec;6(12):3950-4; and Nijhuis et al., Room-temperature single-electron tunneling in dendrimer-stabilized gold nanoparticles anchored at a molecular printboard, Small. 2006 Dec;2(12):1422-6.

Exemplary methods for administering such compounds will be apparent to the skilled artisan. The usefulness of these formulations may depend on the particular composition used and the particular subject receiving the treatment. These formulations may contain a liquid carrier that may be oily, aqueous, emulsified or contain certain solvents suitable to the mode of administration.

The compositions can be administered intermittently or at a gradual, continuous, constant or controlled rate to a warm-blooded animal (e.g., a mammal such as a mouse, rat, cat, rabbit, dog, pig, cow, or monkey), but advantageously are administered to a human being. In addition, the time of day and the number of times per day that the pharmaceutical formulation is administered can vary.

Preferably, the compositions are administered such that active ingredients interact with regions where cancer cells are located. The compounds described herein are very potent at treating these cancers.

In certain circumstances, the compounds described herein can be employed as part of a pharmaceutical composition with other compounds intended to prevent or treat a particular cancer, i.e., combination therapy. In addition to effective amounts of the compounds described herein, the pharmaceutical compositions can also include various other components as additives or adjuncts.

Combination Therapy

The combination therapy may be administered as (a) a single pharmaceutical composition which comprises a selective TrkA agonist or partial agonist as described herein, for example, gambogic amide, at least one additional pharmaceutical agent described herein, and a pharmaceutically acceptable excipient, diluent, or carrier; or (b) two separate pharmaceutical compositions comprising (i) a first composition comprising a selective TrkA agonist or partial agonist as described herein, for example, gambogic amide, and a pharmaceutically acceptable excipient, diluent, or carrier, and (ii) a second composition comprising at least one additional pharmaceutical agent described herein and a pharmaceutically acceptable excipient, diluent, or carrier. The pharmaceutical compositions can be administered simultaneously or sequentially and in any order.

When used to treat demyelination disorders such as MS, the selective TrkA agonist or partial agonist can be administered with other compounds known to treat MS, such as interferon and pegylated interferon (Pegasys).

When used to treat or prevent neurodegenerative disorders such as AD and Parkinson's disease, the selective TrkA agonist or partial agonist can be administered with other compounds known to treat such disorders, such as dopamine and Aricept®.

When used to provide neuroprotection, the selective TrkA agonist or partial agonist can be administered with other compounds known to provide neuroprotection, such as adenosine (Dall'lgna et al., "Neuroprotection by caffeine and adenosine $A_{2A}$ receptor blockade of -amyloid neurotoxicity," *British Journal of Pharmacology* (2003) **138**, 1207–1209).

The following examples are illustrative, but not limiting, of the method and compositions of the present invention. Other suitable modifications and adaptations of the variety of conditions and parameters normally encountered in clinical therapy, and which are obvious to those skilled in the art, are within the spirit and scope of the invention.

**Example 1: Gambogic Acid**

Procedure 1:

Step A.

The dry gamboge powder (140 g) was extracted with MeOH (3.times.600 mL) at room temperature for 1 week, after filtration, the solvent was removed under reduced pressure, gave crude extract (122 g) as yellow powder.

Step B. Gambogic Acid Pyridine Salt.

The above crude extract (120 g) was dissolved in pyridine (120 mL), then warm water (30 mL) was added to the stirred solution. After cooling to r.t., some precipitate was observed. Hexane (120 mL) was added to the mixture and the mixture was filtered and the solid was washed with hexane and dried. The salt was purified by

repeated recrystallization from ethanol and gave gambogic acid pyridine salt ($7_5$ g); HPLC: 99%.

Step C. Gambogic Acid.

The gambogic acid pyridine salt (0.4 g) was dissolved in ether (25 mL) and shaken with aqeuous HCl (1N, 25 mL) for 1 h. The ether solution was then washed with water (2.times$_1$0 mL), dried and evaporated to give the title compound (345 mg); HPLC: 99%. $^1$ H NMR ($CDCl_3$): 12.66 (s, 1H), 7.43 (d, J=6.9 Hz, 1H), 6.48 (d, J=10.2 Hz, 1H), 5.97 (t, J=$7_5$ Hz, 1H), 5.26 (d, J=9.9 Hz, 1H), 4.91 (m, 2H), 3.37 (m, 1H), 3.24-2.98 (m, 2H), 2.81 (d, J=6.6 Hz, 1H), 2.41 (d, J=9 Hz, 1H), 2.20 (m, $J_1$ =8.4 Hz, $J_2$ =$5_1$ HZ, 1H), 1.88 (m, 1H), 1.63 (s, 3H), 1.60 (s, 3H), $1_5$8 (s, 3H), $1_5$3 (s, 3H), $1_5$1 (s, 3H), 1.43 (s, 3H), 1.26 (s, 3H), $1_1$8 (s, 3H). MS: 627 (M-H).

Procedure 2:

The crude extract of gamboge (300 mg) was purified by repeated column chromatography ($SiO_2$, hexane-EtOAc gradient) using a Combi Flash SG 100 separation system, gave 18 mg of gambogic acid; HPLC: 94%, MS. 627 (M-H).

**Example 2: A cell-based screen for protecting TrkA expressing cells from apoptosis**

In order to identify small molecules that mimic NGF and activate TrkA, we developed a cell-based apoptotic assay using a cell permeable fluorescent dye MR(DERD)2, which turns red upon caspase-3 cleavage in apoptotic cells. We utilized a murine cell line T17, which was derived from basal forebrain SN56 cells. T17 cells are TrkA stably transfected SN56 cells. The candidates selectively protecting T17 but not SN56 cells from the first round screen are then subjected to neurite outgrowth assay for the secondary screen. The positive compounds were analyzed for TrkA tyrosine phosphorylation, Akt and MAP kinases signaling cascade activation. The screening strategy scheme is depicted in Figure 1A.

We cultured T17 cells in 96-well plates and preincubated the cells with 10 μM compounds for 30 min, followed by 1 μM staurosporine (STS) treatment for 9 h.

MR(DEVD)2 was introduced to the cells 1 h before examination under fluorescent microscope. The apoptotic cells were red, while live cells had no signal. As a positive control, NGF substantially decreased the red cell numbers compared to DMSO control. Using the caspase-3-activated fluorescent dye as a visual assay, we screened 2000 biologically active compounds from the Spectrum Collection Library.

Thirty-one compounds selectively protected T17 but not SN56 cells from STS-initiated apoptosis, indicating that these compounds might act either directly through TrkA receptor or its downstream signaling effectors. The representative results from the screening are shown (Figure 1B, left panel). Even in the absence of NGF, T17 exhibited a stronger anti-apoptotic effect than its parental SN56 cells, indicating that overexpression of TrkA suppresses caspase-3 activation. NGF treatment further enhanced this effect (Figure 1B, right panel).

**Identification of gambogic acid derivatives as survival enhancers**

In our initial screening, 4 out of 31 compounds are gambogic acid derivatives. The library also contains 3 more gambogic acid derivatives, which are inactive. The chemical structures of the 7 compounds are depicted in Figure 2A. To compare the apoptosis inhibitory activity, we preincubated these gambogic acid derivatives (0.5 μM) with T17 and SN56 cells, followed by 1 μM STS for 9 h. Quantitative analysis of the apoptosis inhibitory activities revealed that all these compounds barely protected SN56 cells from apoptosis. By contrast, gambogic acid, gambogic amide, dimethyl gambogate, and dihydrogambogic acid strongly suppressed apoptosis in T17 cells with protective activities even stronger than NGF. However, tetrahydrogambogic acid and acetyl isogambogic acid slightly blocked STS-initiated apoptosis. Decahydrogambogic acid even increased apoptosis in T17 cells (Figure 2B). These findings suggest that some gambogic acid derivatives can trigger programmed cell death, in greement with a previous report that they possess anti-cancer activity (Kasibhatla et al., 2005). Quantitative analysis of the anti-apoptotic activity reveals that $EC_{50}$ for gambogic amide, gambogic acid, dimethyl gambogic acid, and dihydrogambogic acid on T17 cells are 5, 20, 120 and 200 nM, respectively (Figure 2C).

TrkA is highly expressed in hippocampal neurons (Culmsee et al., 2002; Kume et al., 2000; Zhang et al., 1993). TrkA and p75NTR are up-regulated in

hippocampal and cortical neurons under pathophysiological conditions (Kokaia et al., 1998; Lee et al., 1998). Moreover, neuroprotective effects of NGF in hippocampal and cortical neurons have been demonstrated *in vitro* and *in vivo* (Culmsee et al., 1999; Zhang et al., 1993). To examine whether these compounds could promote neuronal survival, we prepared hippocampal neurons and pretreated the primary neurons with various gambogic acid derivatives for 30 min, followed by 50 μM glutamate treatment for 16 h. Quantitative apoptosis assay with MR(DEVD)2 demonstrated that gambogic acid displayed the same protective effect as the positive control NGF, and gambogic amide exhibited even stronger apoptosis inhibitory activity. Surprisingly, dimethyl gambogate, tetrahydrogambogic acid, acetyl isogambogic acid and decahydrogambogic acid even slightly enhanced apoptosis compared to control, and dihydrogambogic acid weakly protected neurons from glutamate-incurred cell death (Figure 2D).

NGF overexpression decreased infarct volume and neuronal apoptosis in transgenic mice or intraventricular injected mice (Guegan et al., 1998; Luk et al., 2004). NGF also potently protected PC12 cells from apoptosis in an OGD (Oxygen-glucose-deprivation) model (Tabakman et al., 2005). To explore whether gambogic amide exerts any protective effect on hippocampal neurons in OGD, we pretreated the primary preparations with NGF or various compounds 30 min before OGD stimulation. In 3 h, apoptotic analysis showed that gambogic amide exhibited the most potent protective effects (Figure 2E, left panel). Titration assay revealed that it protected neurons in a dose-dependent manner (Figure 2E, right panel). Therefore, gambogic amide selectively protects TrkA expression cells and primary neurons from apoptosis.

**Gambogic amide elicits neurite outgrowth in PC12 cells**

One of most prominent neurotrophic effects of NGF is to trigger neurite outgrowth in neuronal cells and incur differentiation. To explore whether gambogic acid compounds possess this activity, we incubated PC12 cells with 0.5 μM compounds for 5 days. The cell medium containing the compounds was replenished every other day. As expected, NGF elicited pronounced neurite sprouting in PC12 cells after 5 days of treatment. In our study, both gambogic amide and dihydrogambogic acid induced evident neurite outgrowth in PC12 cells, whereas

gambogic acid and dimethyl gambogate failed. The neurite network generated by gambogic amide was much more demonstrable than that generated by dihydrogambogic acid, suggesting gambogic amide might possess stronger neurotrophic activity than the latter (Figure 3A). Dose-dependent assay revealed that 10-50 nM of gambogic amide was sufficient to provoke substantial neurite sprouting in PC12 cells (Figure 3B). Thus, gambogic amide possesses potent neurotrophic activity at a concentration comparable to NGF, and robustly provokes neurite outgrowth.

**Gambogic amide triggers TrkA tyrosine phosphorylation in hippocampal neurons**

NGF binds to receptor TrkA and elicits its dimerization and autophosphorylation on tyrosine residues. Numerous tyrosine residues on TrkA are phosphorylated upon NGF stimulation. For example, Y490 phosphorylation is required for Shc association and activation of MAP kinase signaling cascade. Y751 phosphorylation is essential for PI 3-kinase docking and activation. To evaluate whether gambogic acid compounds could also trigger TrkA tyrosine phosphorylation, we treated primary hippocampal neurons with various drugs at 0.5 μM for 30 min. The cell lysates were analyzed by immunoblotting with anti-phospho-TrkA Y490 antibody. NGF treatment induced potent TrkA phosphorylation. Surprisingly, only gambogic amide but not other compounds elicited TrkA phosphorylation (Figure 4A, top panel). Gambogic amide also initiated the pronounced TrkA tyrosine phosphorylation in hippocampal neurons, as demonstrated by immunofluorescent staining with anti-TrkA Y490 specific antibody (Figure 4B). To explore whether gambogic amide can trigger TrkA dimerization, we cotransfected GFP-TrkA and HA-TrkA into HEK293 cells, and treated the cells with 0.5 μM gambogic amide for 30 min. Coimmunoprecipitation assay demonstrated that gambogic amide provoked TrkA dimerization even more strongly than NGF, whereas gambogic acid and DMSO failed to do so. By contrast, the cotransfected TrkB and TrkA receptors failed to dimerize regardless of NGF or gambogic amide treatment (Figure 4C). Gambogic amide also elicited tyrosine phosphorylation in TrkA but not in TrkB or C receptor. Interestingly, TrkA-KD displayed decreased tyrosine phosphorylation compared to wild-type TrkA (Figure 4D), indicating that not only TrkA autophosphorylation but

also other tyrosine kinases activated by gambogic amide contribute to Y490 phosphorylation. Hence, these results demonstrate that gambogic amide mimics NGF and selectively provokes TrkA dimerization and tyrosine phosphorylation.

**Gambogic amide provokes Akt and MAP kinase activation**

NGF triggers PI 3-kinase/Akt and Ras/MAP Kinase signaling cascades activation through the TrkA receptor. To explore whether the gambogic acid derivatives possess similar mitogenic effects, we treated T17 cells with various gambogic acid derivatives for 30 min. The cell lysates were analyzed by immunoblotting with anti-phospho-Erk1/2 and phospho-Akt-473 antibodies, respectively. NGF treatment stimulated demonstrable Erk1/2 and Akt phosphorylation. Strikingly, gambogic amide provoked robust phosphorylation of both Erk1/2 and Akt, whereas gambogic acid, tetrahydrogambogic acid and decahydrogambogic acid failed to activate MAP kinases. Dimethyl gambogic acid, dihydrogambogic acid and acetyl isogambogic acid weakly agitated MAP kinase activation. Although gambogic acid and decahydrogambogic acid were unable to activate MAP kinase, they strongly provoked Akt activation (Figure 5A), suggesting that these compounds might differentially regulate PI 3-kinase/Akt and Ras/MAP kinase pathways either through TrkA receptor or its downstream cellular targets. We also extended the assays into primary hippocampal neurons. NGF, gambogic acid, gambogic amide and tetrahydrogambogic acid evidently activated Akt, while other gambogic derivatives slightly upregulated Akt phosphorylation (Figure 5B). Time course assay with hippocampal neurons showed that gambogic amide (0.5 μM) elicited Akt phosphorylation after 5 min treatment, and Akt activation increased after 10 min and sustained for 60 min. The signal faded away after 180 min (Figure 5C, left panel). Gambogic amide also provoked Akt activation in a dose-dependent manner. 50 nM gambogic amide induced Akt activation as strongly as 50 ng/ml NGF did (Figure 5C, right panel). Taken together, these observations support the hypothesis that gambogic amide mimics NGF and potently activates Akt and MAP kinases activation in neurons.

**Gambogic acid binds the cytoplasmic juxtamembrane domain of TrkA receptor**

The immunoglobulin (Ig)-like domain (TrkA-d5 domain) in the extracellular region of TrkA proximal to the membrane is required for specific binding of NGF (Urfer et al., 1995). To investigate which portion of TrkA receptor binds to gambogic amide, we conducted *in vitro* binding assay with immobilized gambogic amide through covalent linkage of affi-gel 102. We generated numerous GFP-tagged TrkA truncates and transfected them into HEK293 cells (Figure 6A, upper panel). Binding assay showed that the extracellular domain was not required for the association. Interestingly, the cytoplasmic juxtamembrane domain was critical for the ligand binding (Figure 6B, lower panels). Although the intracellular domain (ICD) of Trk family members shares great homology, the juxtamembrane region varies (Figure 6C, top panel). *In vitro* binding assay revealed that gambogic acid selectively bound to both wild-type and kinase-dead TrkA but not to TrkB or TrkC. Noticeably, Trk-KD exhibited weaker affinity to gambogic amide than did wild-type TrkA (Figure 6C, lower panel). It did not bind to p75NTR, ErbB3 or EGF receptor (data not shown), underscoring the finding that gambogic amide specifically associates with TrkA but not other neurotrophin receptors or transmembrane tyrosine kinase receptors. To determine the binding constant between gambogic acid and TrkA, we conducted a competition assay with GFP-TrkA-bound gambogic amide beads. The beads-associated TrkA was gradually decreased as the free gambogic amide concentrations increased. In contrast, the inactive tetrahydrogambogic acid failed. Quantitative analysis of the competition data revealed the $K_d$ is about 75 nM (Figure 6D). Incubation of FITC-conjugated gambogic amide with PC12 cells for 10 min elicited its tight association with TrkA receptor, whereas FITC alone failed to penetrate into cells (Figure 6E). Thus, gambogic amide penetrates the cell membrane and binds tightly to TrkA receptor through a different region from NGF.

**Gambogic amide prevents Kainic acid-triggered neuronal apoptosis and decreases infarct volume in stroked rat brain**

Kainic acid (KA) is a potent agonist for the AMPA receptor. Peripheral injections of KA result in recurrent seizures and the subsequent degeneration of select populations of neurons in the hippocampus (Nadler et al., 1980; Schauwecker and Steward, 1997; Sperk et al., 1983). It has been shown that the activation of caspase-3 is a necessary component of KA-induced cell death (Faherty et al., 1999). To explore whether gambogic amide can block the neurotoxicity initiated by KA, we

subcutaneously injected 2 mg/kg gambogic amide into C57BL/6 mice, followed by 25 mg/kg KA. In 5 days, the mice were perfused and the brains were cut to a thickness of 5 μm and mounted on slides. TUNEL staining revealed that KA provoked enormous apoptosis in the hippocampus, and this was substantially diminished by gambogic amide (Figure 7A, left panel). Quantitative analysis of apoptosis in the hippocampus revealed that KA induced 47% and 57% cell death in the CA1 and CA3 region, whereas gambogic amide strongly decreased apoptosis to 6.3% and 1.8% in these two regions, respectively (Figure 7A, right panel).

To further determine the neuroprotective potential *in vivo*, gambogic amide was tested in a transient middle cerebral artery occlusion (MCAO) stroke model in adult male rats. After 2 h MCAO followed by reperfusion, the animals received vehicle or gambogic amide (2 mg/kg) 5 min prior to the onset of reperfusion. All animals included in the study survived the ischemic insult and treatment with gambogic amide. A representative brain slice stained with TTC 24 h after MCAO in vehicle-treated and Gambogic amide-treated rats is shown (Fig. 7B, left panel). Area and volume measurements from TTC sections indicated that treatments with GA substantially reduced infarct volumes in this transient ischemic model of stroke (Figure 7B, right panel). These results concur with a previous report that NGF reduces infarct volume and apoptosis in focal ischemia (Guegan et al., 1998). LDF (laser-Doppler flowmetry) was measured over the ipsilateral parietal cortex. Relative CBF (cerebral blood flow) was reduced to 24.41% (± 3.78%) and 25.09% (± 5.73%) within 5 minutes of MCAO in rats that subsequently received GA or vehicle treatment. After filament withdrawal (120 min), relative CBF increased to 85.83% (± 4.94%) in the GA-treated group and to 87.81% (± 2.23%) in the vehicle-treated group compared with preischemic levels. There were no significant differences between groups suggesting that the relative ischemic insult was equivalent among all groups. Taken together, gambogic amide is a potent TrkA agonist, prevents neuronal cell death and protects the neurodegeneration elicited by excitatory neurotoxicity and stroke.

**Discussion**

The present study demonstrates that gambogic amide mimics NGF and acts as a robust TrkA agonist. However, gambogic amide and NGF bind to different regions on TrkA. NGF is a homodimer polypeptide, which associates with the

immunoglobulin-like domain (TrkA-d5 domain) proximal to the membrane, whereas gambogic amide robustly binds the cytoplasmic juxtamembrane region. NGF triggers TrkA activation through promoting its dimerization and autophosphorylation. Gambogic amide but not acid also stimulates TrkA's dimerization and tyrosine phosphorylation (Figure 5). Based on the crystal structure of gambogic acid, the xanthone ring structure is planar and has top and bottom faces. The two prenyl chains and the polycyclic ring on top are hydrophobic, whereas the carboxylic acid and carbonyl in the polycyclic ring on the bottom are hydrophilic (trans-gambogic acid). Presumably, the top hydrophobic prenyl chains and caged polycyclic rings are inserted into the hydrophobic cleft formed by the cytoplasmic juxtamembrane region, whereas the bottom hydrophilic amide group might stick above the surface and bind to hydrophilic side groups from the backbone of TrkA or $H_2O$ on the surface. It remains unknown exactly how gambogic amide provokes TrkA dimerization and autophosphorylation. Presumably, it provokes TrkA conformational change and reduces the autoinhibitory effect by the Ig2 domain, which can block TrkA dimerization in the absence of NGF (Arevalo et al., 2000). It is worth noting that gambogic amide also provokes faint TrkA-KD tyrosine phosphorylation, suggesting that Y490 is not only phosphorylated by the receptor itself, but other tyrosine kinases might also be implicated in this process. Interestingly, gambogic amide but not the acid possesses evident neurotrophic activities. For example, gambogic amide strongly stimulates both Akt and MAP kinase activation and triggers TrkA tyrosine phosphorylation in hippocampal neurons; by contrast, gambogic acid can elicit only Akt but not MAP kinase activation, and fails to induce TrkA tyrosine phosphorylation (Figure 3 and 4). These results are consistent with the observation that gambogic amide but not gambogic acid enhances TrkA dimerization (Figure 5).

The structure-activity study with T17 cells and hippocampal neurons reveals that the carboxyl group can tolerate different modifications, suggesting that the carboxyl side chain might not be critical for binding to its biological targets. Therefore, the carboxyl group can be used to prepare compounds to optimize the apoptosis-inhibitory efficacy. Reduction of the unsaturated prenyl side chains or C9-C10 α,β-unsaturated ketone and C12 ketone (Figure 2A) completely abolishes the protective effect. On the contrary, it even triggers substantial apoptosis in hippocampal neurons. These results demonstrate that the spatial structure of prenyl

side chains and unsaturated double bonds in the caged polycyclic rings are essential for its neurotrophic effects. Notably, some of gambogic acid derivatives exhibit robust anti-apoptotic activity in T17 but not in hippocampal neurons. Probably, this effect is due to TrkA overexpression in T17 cells, since T17 cells display much stronger anti-apoptotic effects compared to their parental SN56 cells even in the absence of any drugs (Figure 2B).

Mounting evidence demonstrates that Trk family members play a crucial role in initiation, progression, and metastasis of many tumors in humans (Descamps et al., 2001; Douma et al., 2004; McGregor et al., 1999). Members of the neurotrophin receptor family are up-regulated in a variety of human cancers, including prostate (Weeraratna et al., 2000), pancreatic (Zhang et al., 2005) and breast cancers. For instance, NGF strongly stimulates breast cancer cell growth, which is mediated by TrkA and p75NTR respectively (Dolle et al., 2004). Emerging evidence demonstrates that TrkA plays a key role in the progression of these cancers. In the case of pancreatic cancer, increased expression of TrkA also correlates with an increased level of pain. Staurosporine Trk inhibitors from Cephalon Pharmaceuticals have shown excellent preclinical anti-tumor efficacy (George et al., 1999) and have entered human clinical trials (Lippa et al., 2006; Marshall et al., 2005. Since TrkA abnormal activation in numerous tumors contributes to cancer progression, small molecules like gambogic amide might not only prevent neuronal cells from death, they might also promote cancer proliferation as well).

Gambogic acid has been used in traditional Chinese medicine to treat cancers. It potently blocks human cancer proliferation *in vitro* and in animals (Wu et al., 2004; Zhang et al., 2004; Zhao et al., 2004). Recently, it has been shown that the Transferrin receptor functions as a cellular target for gambogic acid to exert its anticancer activity. Presumably, gambogic amide, the most potent TrkA agonist we identified, might bind Transferrin receptor as well as TrkA. Gambogic acid associates with Transferrin with $K_d$ of 2.2 μM (Kasibhatla et al., 2005). Conceivably, gambogic amide binds to TrkA receptor much more specifically and tightly than to Transferrin receptor. Consequently, it might exert neurotrophic activity more robustly and selectively than apoptotic effects. NGF regulates neuronal apoptosis through the action of critical protein kinase cascades, such as the phosphoinositide 3-kinase/Akt and mitogen-activated protein kinase pathways. Since neurodegeneration is an

underlying cause of various nervous system disorders, including Alzheimer's disease and amyotrophic lateral sclerosis, it is important that molecules, which provide trophic support for neurons, are identified, and their mechanisms of action defined. In addition to its role as target-derived survival factors, NGF also modulates activity-dependent neuronal plasticity in adult neurons. Moreover, NGF may be useful for the treatment of neurodegenerative disorders such as Alzheimer's disease (Olson, 1993). Thus, our findings that gambogic amide is a novel TrkA agonist and prevents neuronal cell death may be clinically important for the treatment of various neurodegenerative diseases and stroke.

**Materials and methods**

**Cells and reagents**

PC12 cells were maintained in medium A (DMEM with 10% fetal bovine serum (FBS), 5% horse serum and 100 units penicillin-streptomycin) at 37°C with 5% $CO_2$ atmosphere in a humidified incubator. Mouse septal neuron x neuroblastoma hybrids SN56 cells were created by fusing N18TG2 neuroblastoma cells with murine (strain C57BL/6) neurons from postnatal 21 days septa. SN56 cells were maintained at 37°C with 5% $CO_2$ atmosphere in DMEM medium containing 1 mM pyruvate and 10% FBS. T17 cells, stably transfected with rat TrkA were cultured in the same medium containing 300 μg/ml G418. The cells are gifts from Dr. Brygida Berse at Boston University. NGF was from Roche. Phospho-Akt-473 or 308, Akt and lamin A/C antibodies were from Cell Signaling. Anti-phospho-Erk1/2, anti-phospho-TrkA Y490, and anti-phospho-Akt 473 antibodies were from Upstate Biotechnology, Inc. The chemical library containing 2000 biologically active compounds was from The Spectrum Collection (MicroSource Discovery System, Inc. Gaylordsville, CT 06755). All chemicals not included above were purchased from Sigma.

**Cell-based Screen**

T17 cells were seeded in a 96-well plate at 10,000 cells/well in 100 μl complete medium. Cells were incubated overnight, followed by 30 min pretreatment with 10 μM compounds in DMSO (10 mM stock concentration from The Spectrum Collection library). The cells were then treated with 1 μM Staurosporine for 9 h. One h before the termination of the experiment, 10 μM MR(DEVD)2, a cell permeable

caspase-3-activated fluorescent dye was introduced. Cells were fixed with 4% paraformaldehyde for 15 min. Cells were washed with PBS and incubated with 1 μg/ml of Hoechst 33342 for 10 min. Cover slides were washed with PBS, mounted, and examined using a fluorescence microscope.

**Immobilization of gambogic acid and synthesis of FITC-gambogic acid**

Gambogic acid was immobilized to affi-gel 102 (Bio-Rad), which contains free amine group, using EDC and NHS. The amidation reaction was carried out at room temperature. In brief, a mixture of reduced gambogic acid (6.288 mg, 10 μmole), NHS (1.1 mg, 5 mmole), EDC (0.4 mg, 2 mmole) and 1 ml affi-gel 102 in ethanol (1 mL) was stirred at room temperature for 5 h. The reaction mixture was washed 3 times with large volume of 100% ethanol. The gambogic acid-conjugated beads were kept in 1 X PBS at 4 °C in dark. For synthesizing FITC-conjugated gambogic acid, gambogic acid was activated first with EDC and NHS in ethanol. The reaction mixture was introduced into 1 ml FITC in ethanol (6.53 mg, 10 μmole). After 2 h incubation at room temperature, the reaction solution was poured into water (10 mL), then extracted with ethyl acetate (3 x 10 mL). The combined organic layer was dried and concentrated to yield the crude product, which was purified by chromatography (SiO2, EtOAc–hexane) to yield the desired compounds.

**Co-immunoprecipitation and *In Vitro* Binding Assays**

A 10-cm plate of HEK293 cells or PC12 cells were washed once in PBS, and lysed in 1 ml lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1.5 mM $Na_3VO_4$, 50 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate, 1 mM phenylmethylsulfonyl flouride (PMSF), 5 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin A), and centrifuged for 10 min at 14,000 x g at 4 °C. The supernatant was transferred to a fresh tube. After SDS-PAGE, the samples were transferred to a nitrocellular membrane. Western blotting analysis was performed with a variety of antibody.

**Kainic Acid/Gambogic amide Drug Administration**

Male C57BL/6 mice aged of 60 days were injected subcutaneously (s.c.) with a single dose of either 30% ethanol in saline or KA (25 mg/kg) (Sigma, MO) or gambogic amide (2 mg/kg) followed by KA. Animals were continually monitored for

2 h for the onset of seizure activity. At 5 days following treatment, animals were anesthetized and perfused with 4% paraformaldehyde in 0.1 M phosphate buffered saline. Brains were removed, post-fixed overnight and processed for paraffin embedding. Serial sections were cut at 5 μm and mounted on slides (Superfrost-plus, Fisher). The slides were processed for TUNEL staining in order to assess the degree of DNA fragmentation.

**Focal ischemia model**

A total of 10 rats were used in the present study; 2 rats were excluded because of inadequate occlusion and reperfusion. Criteria for inclusion/exclusion of rats from the study group were based on the laser-Doppler flowmetry (LDF) measurement of cerebral blood flow (CBF). To ensure relative uniformity of the ischemic insult, animals with mean ischemic LDF >40% of baseline LDF were excluded from the cohort. This procedure resulted in consistently larger and more uniform infarcts, reducing experimental variablity. Anesthesia was induced by inhalation of 5% isoflurane (in a $N_2/O_2$ 70%/30% mixture) and maintained by inhalation of 2% isoflurane. Using a SurgiVet (model V3304; Waukesha, WI, USA) pulse oximeter, blood $SpO_2$ was monitored and maintained at levels ≥90%. Body temperature was monitored throughout surgery (via rectal probe) and maintained at 36.5° C to 37.5° C using a heating blanket (Harvard Apparatus, South Natick, MA, USA). A small incision was made in the skin overlying the temporalis muscle and the laser-Doppler probe (Moor Instruments, Wilmington, Delaware, USA) was positioned on the superior portion of the temporal bone (6 mm lateral and 2 mm posterior from bregma). Focal cerebral ischemia was induced by occlusion of the right middle cerebral artery as previously described (Sayeed et al., 2006). ***Lesion Volume:*** The rats were sacrificed 24 h post-occlusion with an overdose (75 mg/kg) of Nembutal sodium solution. The brains were carefully removed and placed in ice-cold saline, and then sliced into 7 serial coronal sections of 2 mm thickness with a rat brain matrix (Harvard Apparatus) starting at 1 mm posterior to the anterior pole. After sectioning, the slices were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma) in saline and kept for 10 min at 37°C in the dark. Stained sections were then fixed in 10% buffered formalin. Both hemispheres of each stained coronal section were scanned using a high-resolution scanner (Epson Perfection 2400 Photo), and then

evaluated by digital image analysis (Image Pro System, Media Cybernetics, Silver Spring, MD, USA). ***Drug Administration:*** The rats subjected to MCAO incurring ischemic insult <40% of baseline LDF were randomly assigned to receive either GA (n=4), or vehicle (n=4) treatment. GA was given in the amount of 2 mg/kg by ip injection 5 min prior to the onset of reperfusion. Rats in the vehicle group underwent the same experimental protocol, except that they received an identical volume/weight of vehicle only.

**Statistical analysis:** All results were expressed as mean ± SD. Mean ischemic LDF and lesion volume were analyzed using the Student's t-test. The criterion for statistical significance was set at $p<0.05$.

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Having now fully described this invention, it will be understood by those of ordinary skill in the art that the same can be performed within a wide and equivalent range of conditions, formulations and other parameters without affecting the scope of the invention or any embodiment thereof. All patents, patent applications and publications cited herein are fully incorporated by reference herein in their entirety.

**Claims**

1. An amide analogue of gambogic acid, where the amide group has the formula (CONHR), where R is is hydrogen, optionally substituted lower alkyl, optionally substituted aryl, optionally substituted lower aralkyl groups,

where the substituents are one or more substituents selected from the group consisting of halo, hydroxy, carboxyl, alkoxycarbonyl, amino, nitro, cyano, $C_1$ -$C_6$ acylamino, $C_1$ -$C_6$ aminoacyl, $C_1$ -$C_6$ acyloxy, $C_1$ -$C_6$ alkoxy, aryloxy, alkylthio, $C_6$ -$C_10$ aryl, $C_d$ -$C_7$ cycloalkyl, $C_2$ -$C_6$ alkenyl, $C_2$ -$C_6$ alkynyl, $C_6$ -$C_10$ aryl($C_2$ -$C_6$)alkenyl, $C_6$ -$C_10$ aryl($C_2$ -$C_6$)alkynyl, saturated or partially saturated 5-7 membered heterocyclo group, or heteroaryl,

or a pharmaceutically acceptable salt or prodrug thereof.

2. An amide analogue of gambogic acid, where the amide group has the formula ($CONH_2$), or a pharmaceutically acceptable salt or prodrug thereof.

3. A pharmaceutical composition comprising the compound of any of claims 1 or 2, and a pharmaceutically acceptable carrier.

4. The pharmaceutical composition of Claim 3, further comprising a compound other than an amide analogue of gambogic acid, which compound has neuroprotective activity, myelin protecting activity, or modulates the release of a neurotransmitter.

5. A method of treating or preventing a neurodegenerative disorder responsive to the agonism of TrkA in an mammal, comprising administering to a mammal in need of such treatment an effective amount of a compound of any of claims 1 or 2, or a pharmaceutical composition of Claims 3 or 4.

6. The method of Claim 5, wherein the disorder is stroke, Parkinson's Disease, mild cognitive impairment, Alzheimer's disease, epilepsy, ALS, and other neurodegenerative diseases.

8. A method of providing neuroprotection either before, during, or after an ischemic event, comprising administering a compound of any of claims 1 or 2, or a pharmaceutical composition of Claims 3 or 4, to a patient in need of neuroprotection.

9. The method of Claim 8, wherein the compound or composition is administered following a stroke or other ischemic event.

10. The method of Claim 8, wherein the compound or composition is administered following exposure to a neurotoxin.

11. The method according to claim 8, wherein said compound or composition is administered together with at least one known neuroprotective agent, or a pharmaceutically acceptable salt of the agent.

12. A method of treating or preventing a demyelinating disorder, comprising administering a compound of any of claims 1 or 2, or a pharmaceutical composition of Claims 3 or 4, to a patient in need of treatment or prevention thereof.

13. The method of Claim 12, wherein the demyelinating disorder is multiple sclerosis.

14. The method of Claim 12, wherein the compound or composition is administered together with interferon or a pegylated version thereof.

15. A method for identifying selective TrkA agonists or partial agonists, comprising:

a) obtaining a library of putative TrkA agonists or partial agonists,

b) subjecting the compounds in the library to a cell-based apoptotic assay that compares the ability of a compound with the ability of nerve growth factor (NGF) to protect cells expressing TrkA from apoptosis,

wherein the assay uses a cell permeable fluorescent dye, such as MR(DERD)2, which turns red upon caspase-3 cleavage in apoptotic cells,

the cells used in the assay are from a murine cell line T17, which was derived from basal forebrain SN56 cells, and

the T17 cells are TrkA stably transfected SN56 cells, and

wherein compounds that protect these cells from apoptosis are either TrkA agonists or partial agonists, or affect downstream effectors.

16. The method of Claim 15, further comprising determining which compounds which protect cells from apoptosis also cause TrkA to dimerize.

17. The method of Claim 16, wherein the process for determining which compounds cause TrkA to dimerize involves exposing compounds which protect cells from apoptosis to HEK293 cells which have been co-transfected with GFP-TrkA and HA-TrkA, and observing the presence or absence of TrkA dimerization.

18. The method of Claim 16, further comprising determining which compounds which cause TrkA dimerization are selective TrkA agonists by

determining whether the compounds bind selectively to TrkA in preference to TrkB or TrkC.

19. The method of Claim 18, wherein the method of determining whether a compound is selective for TrkA comprises exposing the compound to HEK293 cells that are co-transfected with TrkA and either or both of TrkB and TrkC receptors,

wherein compounds that are selective for TrkA will fail to dimerize the co-transfected cells.

20. The method of Claim 16, further comprising determining which compounds which cause TrkA dimerization are selective TrkA agonists by determining whether the compounds elicit tyrosine phosphorylation in TrkA, but not in TrkB or C receptors, or in cells co-transfected to express two or more of these receptors.

21. The method of any of claims 15-20, further comprising assessing the ability of one or more selective TrkA agonists or partial agonists identified using these methods to provoke neurite outgrowth in PC12 cells, and/or prevent neuronal cell death.

22. Use of a compound of any of Claims 1 or 2 in the preparation of a medicament for treating or preventing a neurodegenerative disorder responsive to the agonism of TrkA in an mammal.

23. The use of Claim 22, wherein the disorder is stroke, Parkinson's Disease, mild cognitive impairment, Alzheimer's disease, epilepsy, ALS, and other neurodegenerative diseases.

24. Use of a compound of any of Claims 1 or 2 in the preparation of a medicament for providing neuroprotection either before, during, or after an ischemic event.

25. The use of Claim 24, wherein the medicament is administered following a stroke or other ischemic event.

26. The use of Claim 24, wherein the medicament is administered following exposure to a neurotoxin.

27. The use according to claim 24, wherein said medicament is administered together with at least one known neuroprotective agent, or a pharmaceutically acceptable salt of the agent.

28. Use of a compound of any of Claims 1 or 2 in the preparation of a medicament for treating or preventing a demyelinating disorder.

29. The use of Claim 28, wherein the demyelinating disorder is multiple sclerosis.

30. The use of Claim 28, wherein the compound is administered together with interferon or a pegylated version thereof.

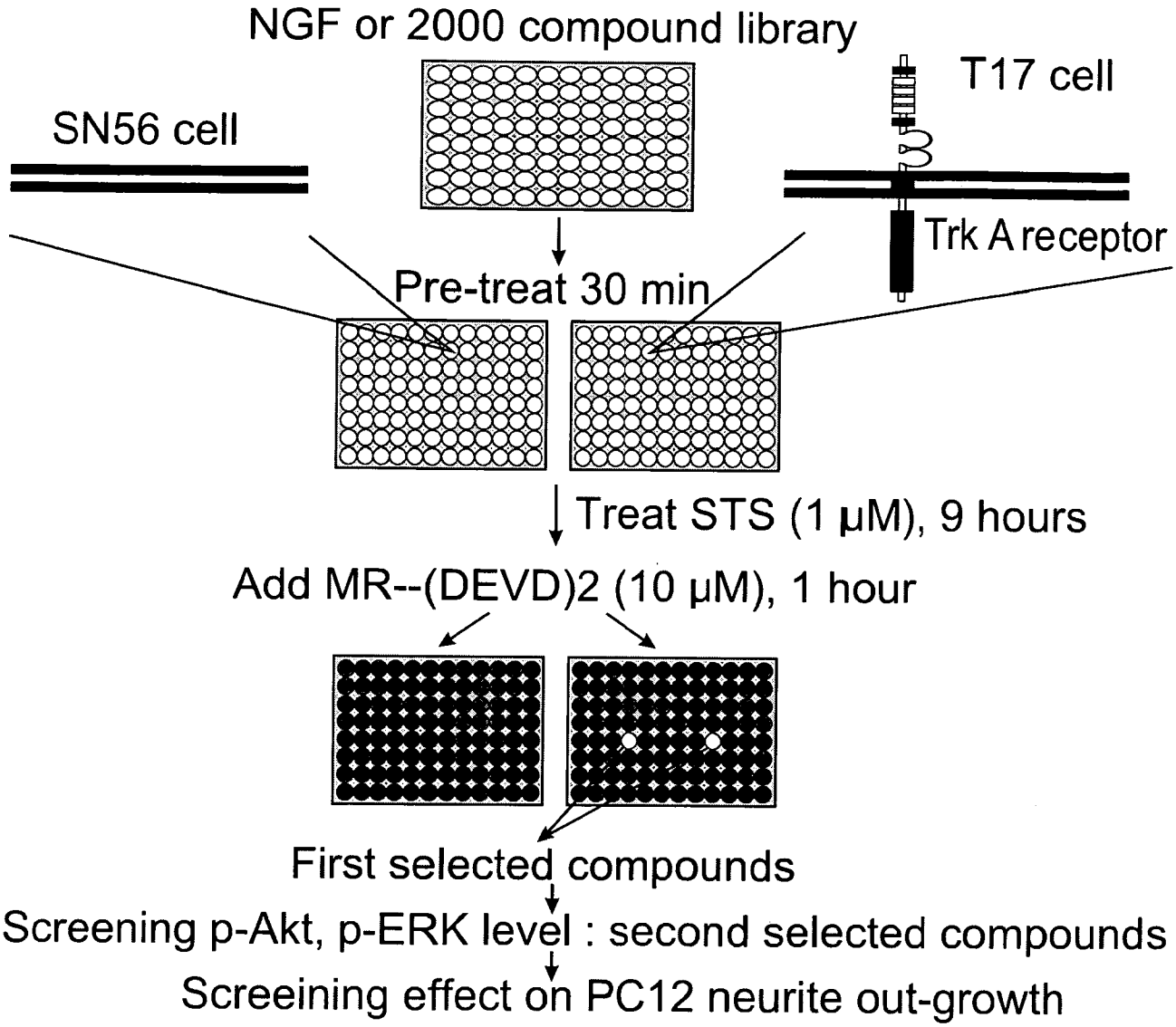

NGF or 2000 compound library
SN56 cell
T17 cell
Trk A receptor
Pre-treat 30 min
Treat STS (1 μM), 9 hours
Add MR--(DEVD)2 (10 μM), 1 hour
First selected compounds
Screening p-Akt, p-ERK level : second selected compounds
Screeining effect on PC12 neurite out-growth

FIG.1A

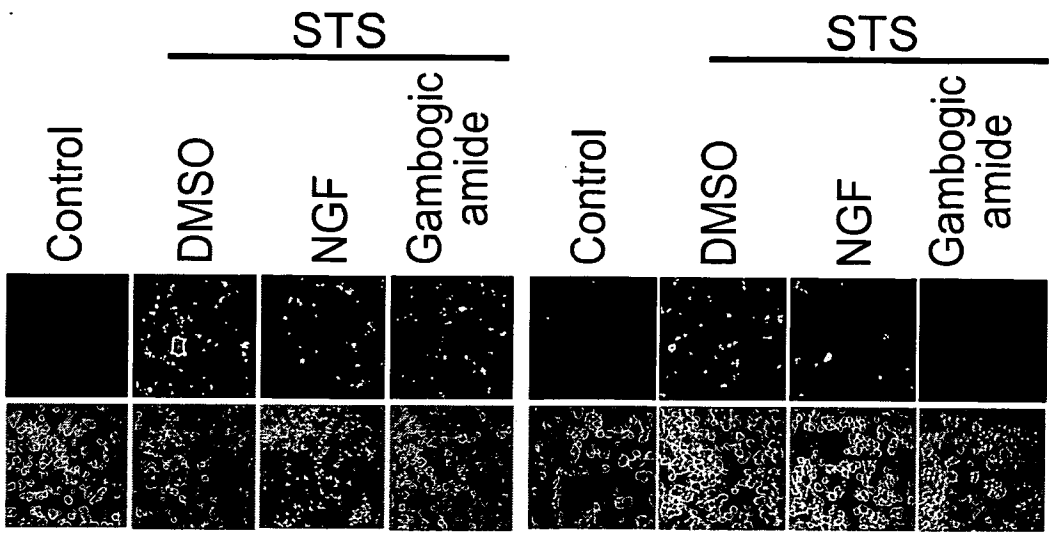
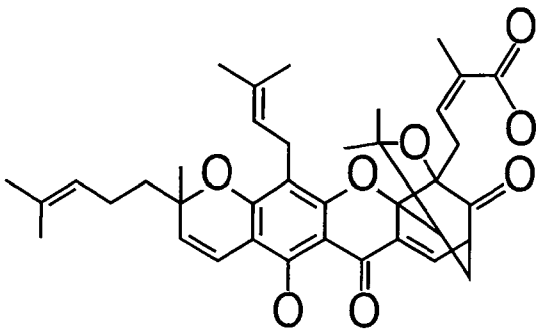
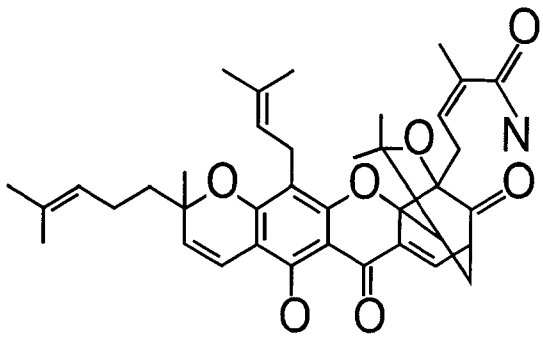

STS
STS
Control
DMSO
NGF
Gambogic amide
Control
DMSO
NGF
Gambogic amide
Sn56 cells
T17 cells

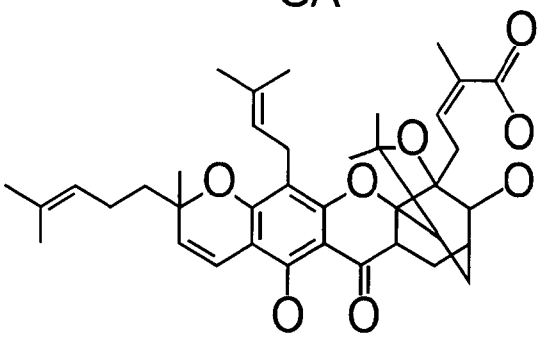
FIG.1B



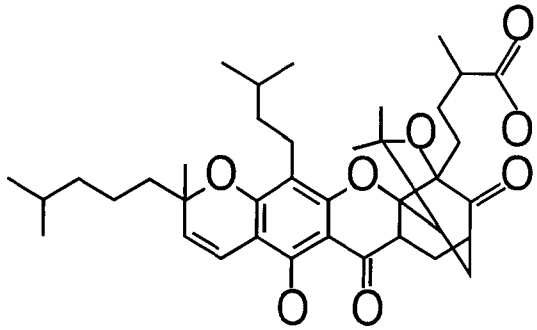
GA



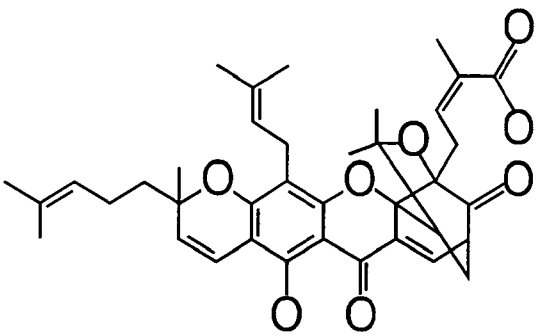
GA-AMIDE



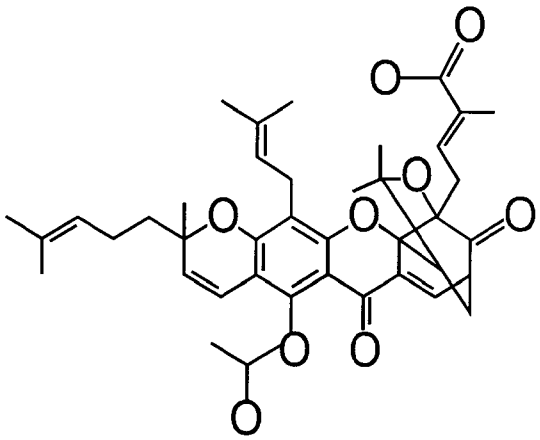
TETRAHYDRO-GA



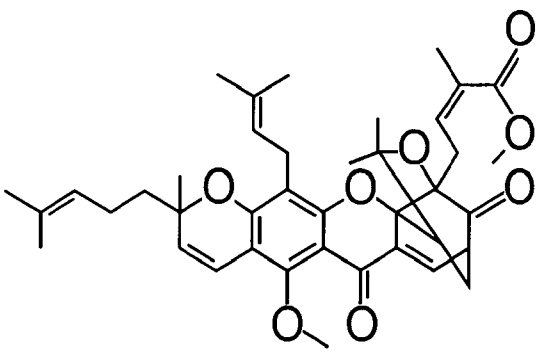
DECAHYDRO-GA



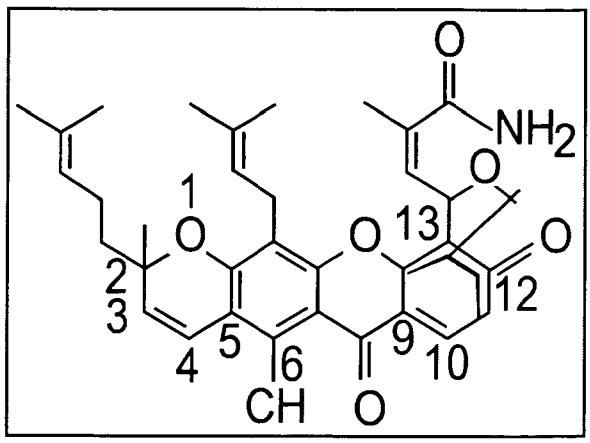
DIHYDRO-GA



ACETYL ISO-GA



DIMETHYL-GA




Gambogic amide

**FIG.2A**

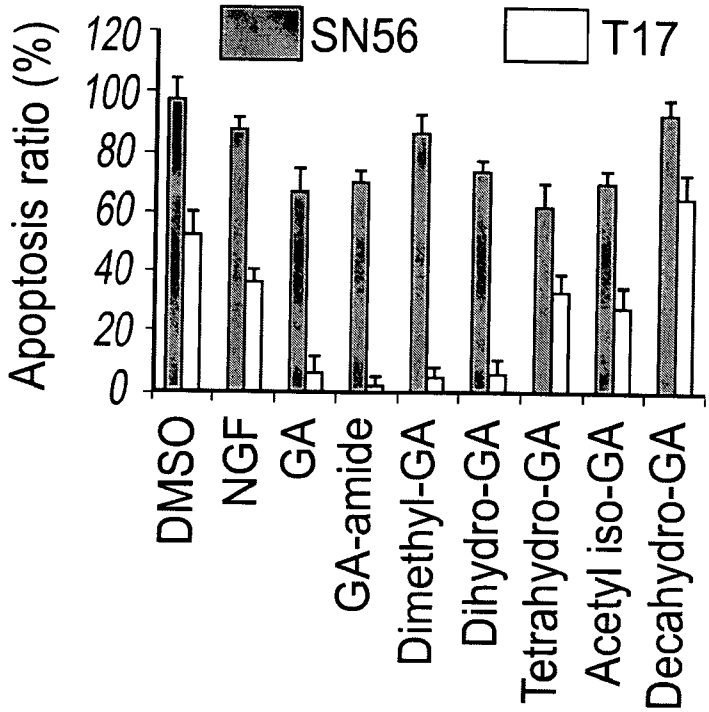

SN56
T17
Apoptosis ratio (%)
120
100
80
60
40
20
0
DMSO
NGF
GA
GA-amide
Dimethyl-GA
Dihydro-GA
Tetrahydro-GA
Acetyl iso-GA
Decahydro-GA

FIG.2B

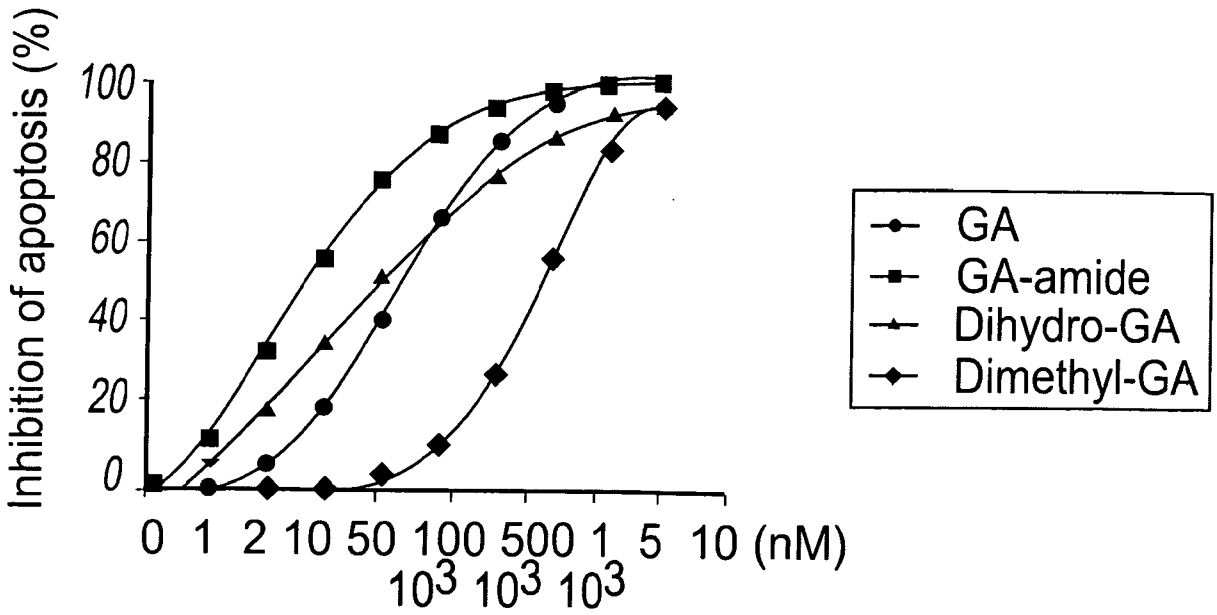

Inhibition of apoptosis (%)
100
80
60
40
20
0
0 1 2 10 50 100 500 1 5 10 (nM)
10³ 10³ 10³
GA
GA-amide
Dihydro-GA
Dimethyl-GA

FIG.2C

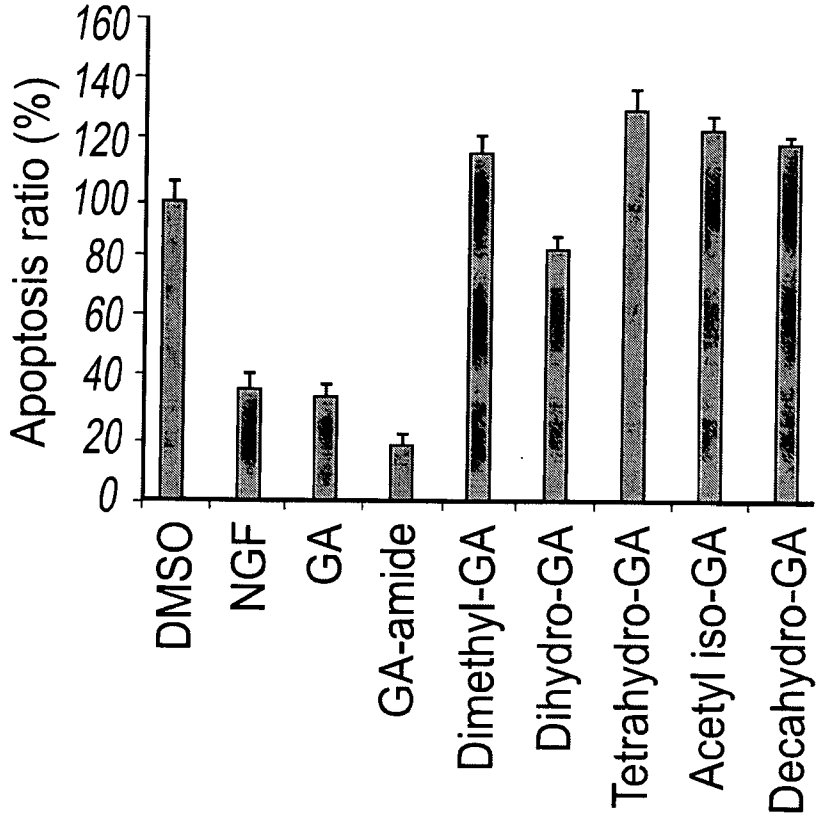

Apoptosis ratio (%)
160
140
120
100
80
60
40
20
0
DMSO
NGF
GA
GA-amide
Dimethyl-GA
Dihydro-GA
Tetrahydro-GA
Acetyl iso-GA
Decahydro-GA

FIG.2D

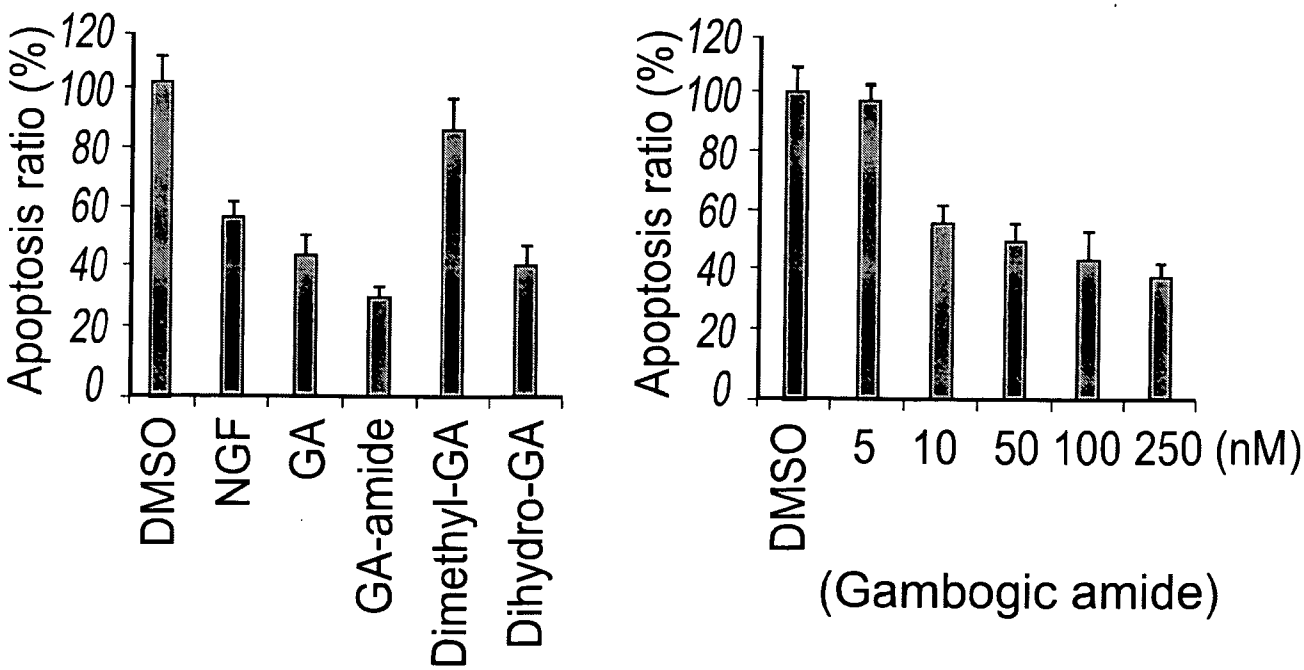

Apoptosis ratio (%)
120
100
80
60
40
20
0
DMSO
NGF
GA
GA-amide
Dimethyl-GA
Dihydro-GA
DMSO
5
10
50
100
250
(nM)
(Gambogic amide)

FIG.2E

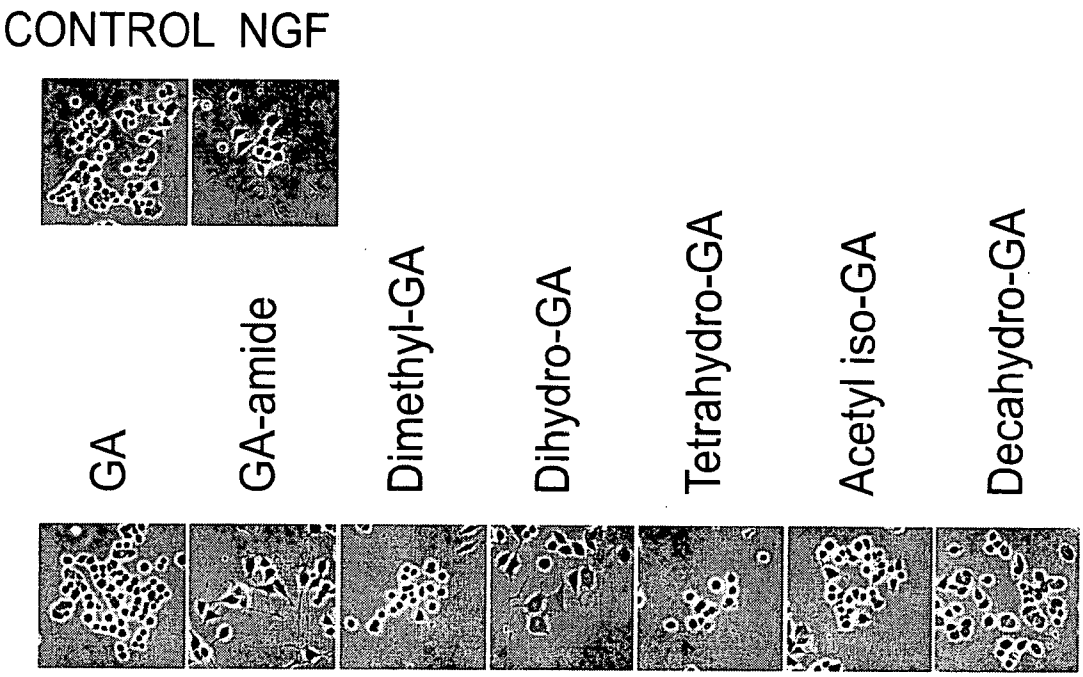

CONTROL NGF
GA
GA-amide
Dimethyl-GA
Dihydro-GA
Tetrahydro-GA
Acetyl iso-GA
Decahydro-GA

FIG.3A

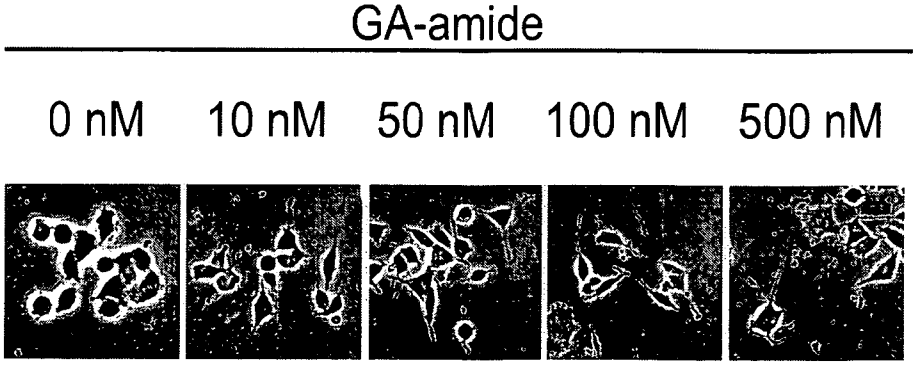

GA-amide
0 nM
10 nM
50 nM
100 nM
500 nM

FIG.3B

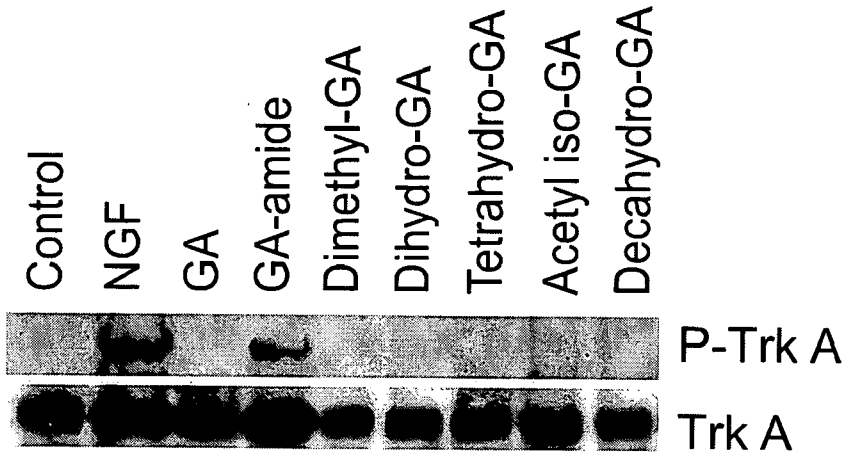

Control
NGF
GA
GA-amide
Dimethyl-GA
Dihydro-GA
Tetrahydro-GA
Acetyl iso-GA
Decahydro-GA
P-Trk A
Trk A

FIG.4A

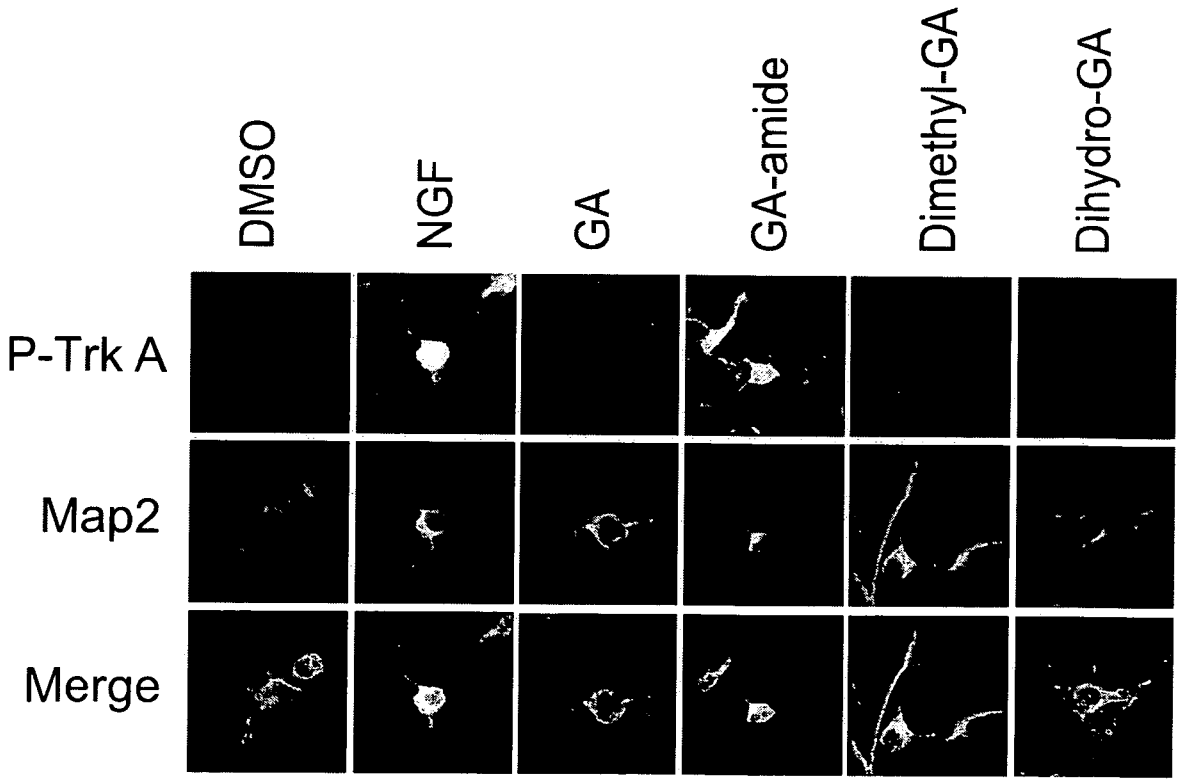

DMSO
NGF
GA
GA-amide
Dimethyl-GA
Dihydro-GA
P-Trk A
Map2
Merge

FIG.4B

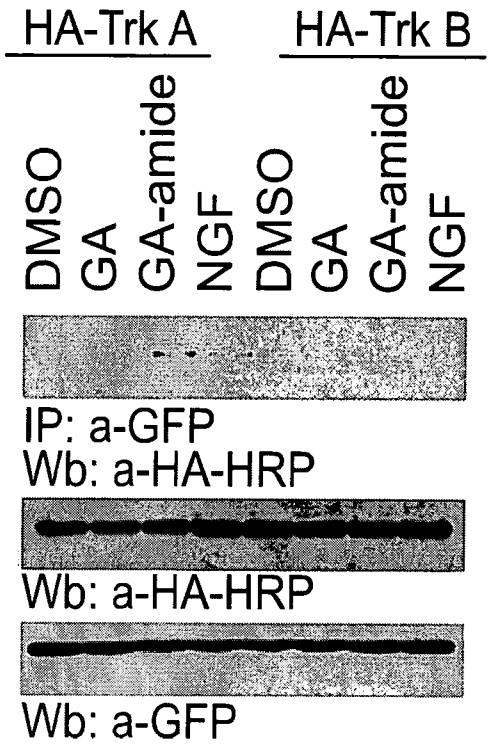

HA-Trk A
HA-Trk B
DMSO
GA
GA-amide
NGF
DMSO
GA
GA-amide
NGF
IP: a-GFP
Wb: a-HA-HRP
Wb: a-HA-HRP
Wb: a-GFP

FIG.4C

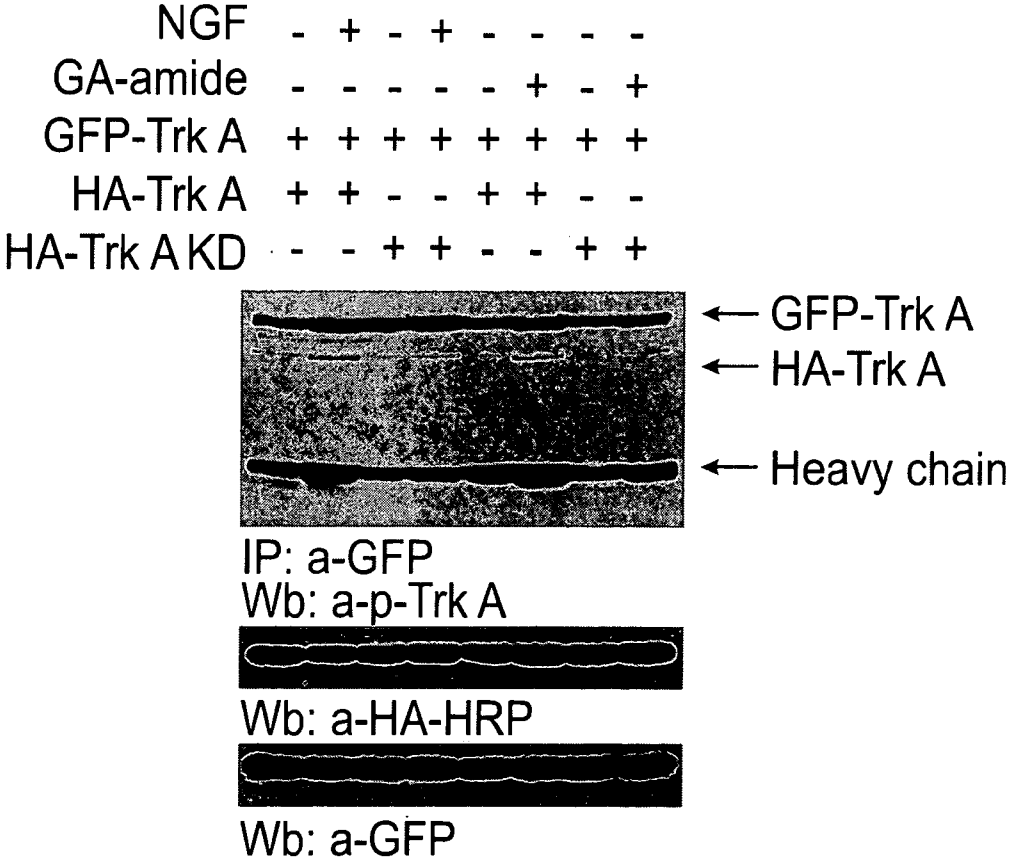

NGF - + - + - - - -
GA-amide - - - - - + - +
GFP-Trk A + + + + + + + +
HA-Trk A + + - - + + - -
HA-Trk A KD - - + + - - + +
GFP-Trk A
HA-Trk A
Heavy chain
IP: a-GFP
Wb: a-p-Trk A
Wb: a-HA-HRP
Wb: a-GFP

FIG.4D

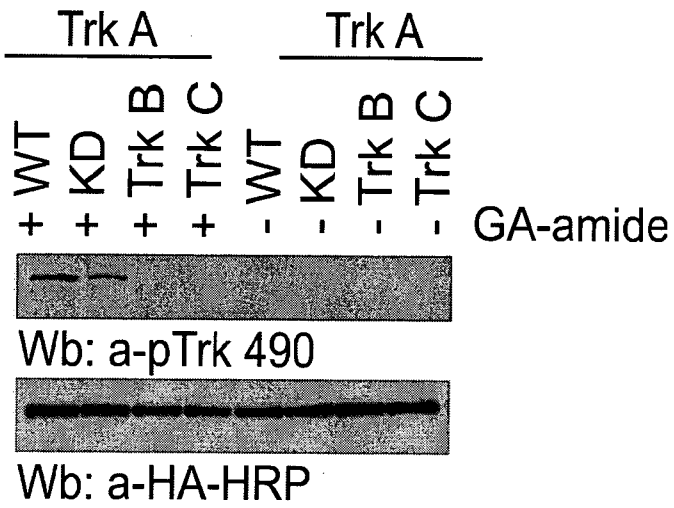

Trk A
Trk A
+ WT
+ KD
+ Trk B
+ Trk C
- WT
- KD
- Trk B
- Trk C
GA-amide
Wb: a-pTrk 490
Wb: a-HA-HRP

FIG.4E

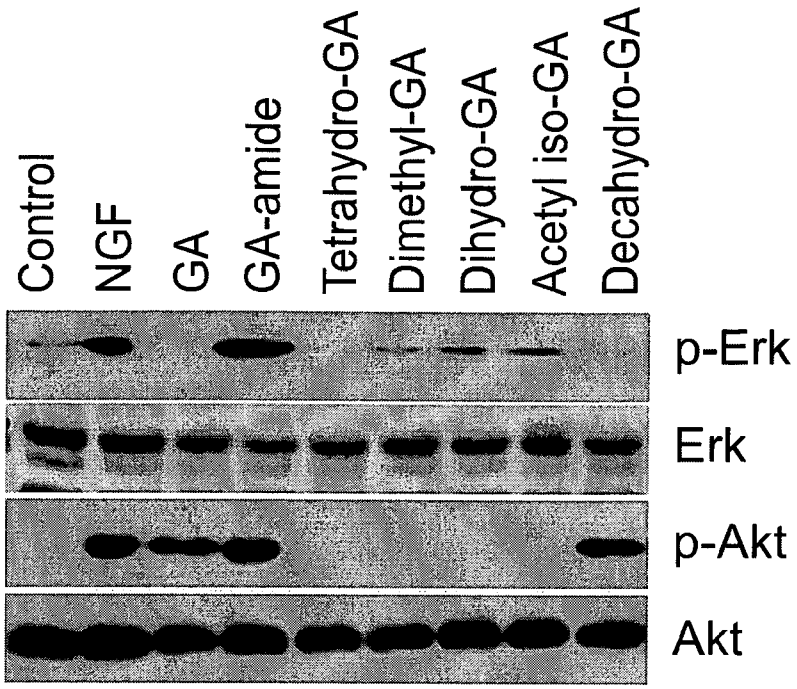



FIG.5A

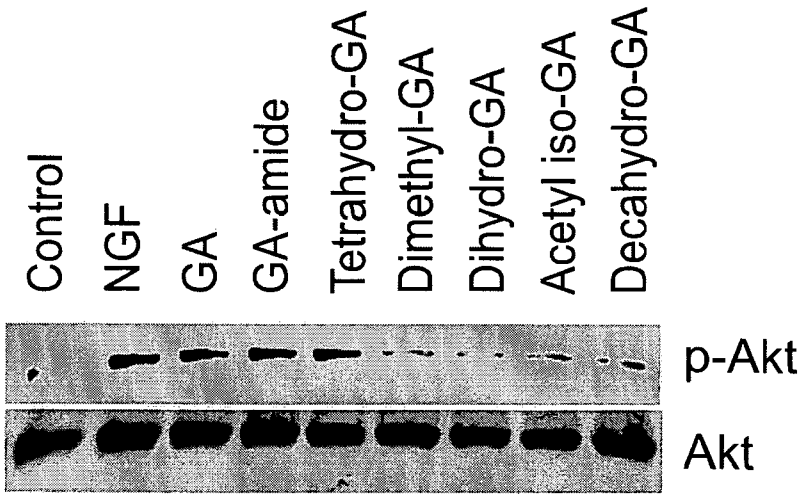



FIG.5B

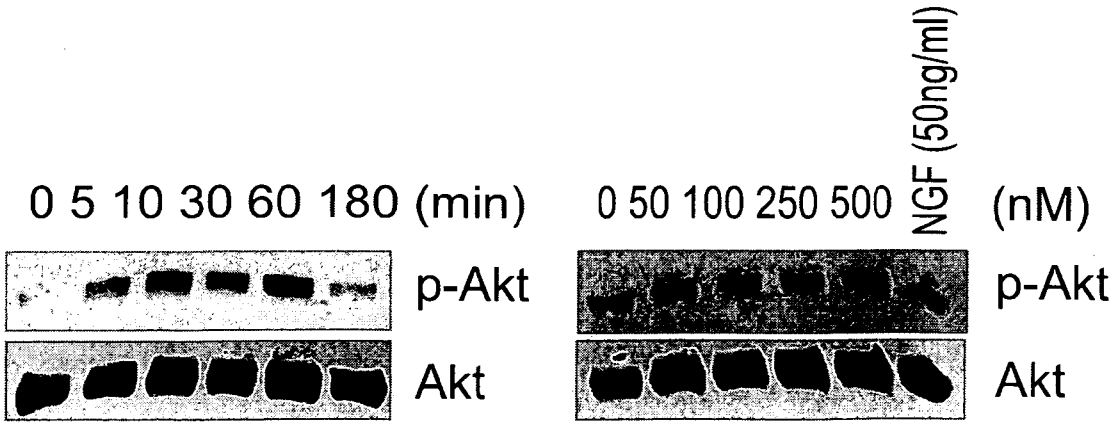



FIG.5C

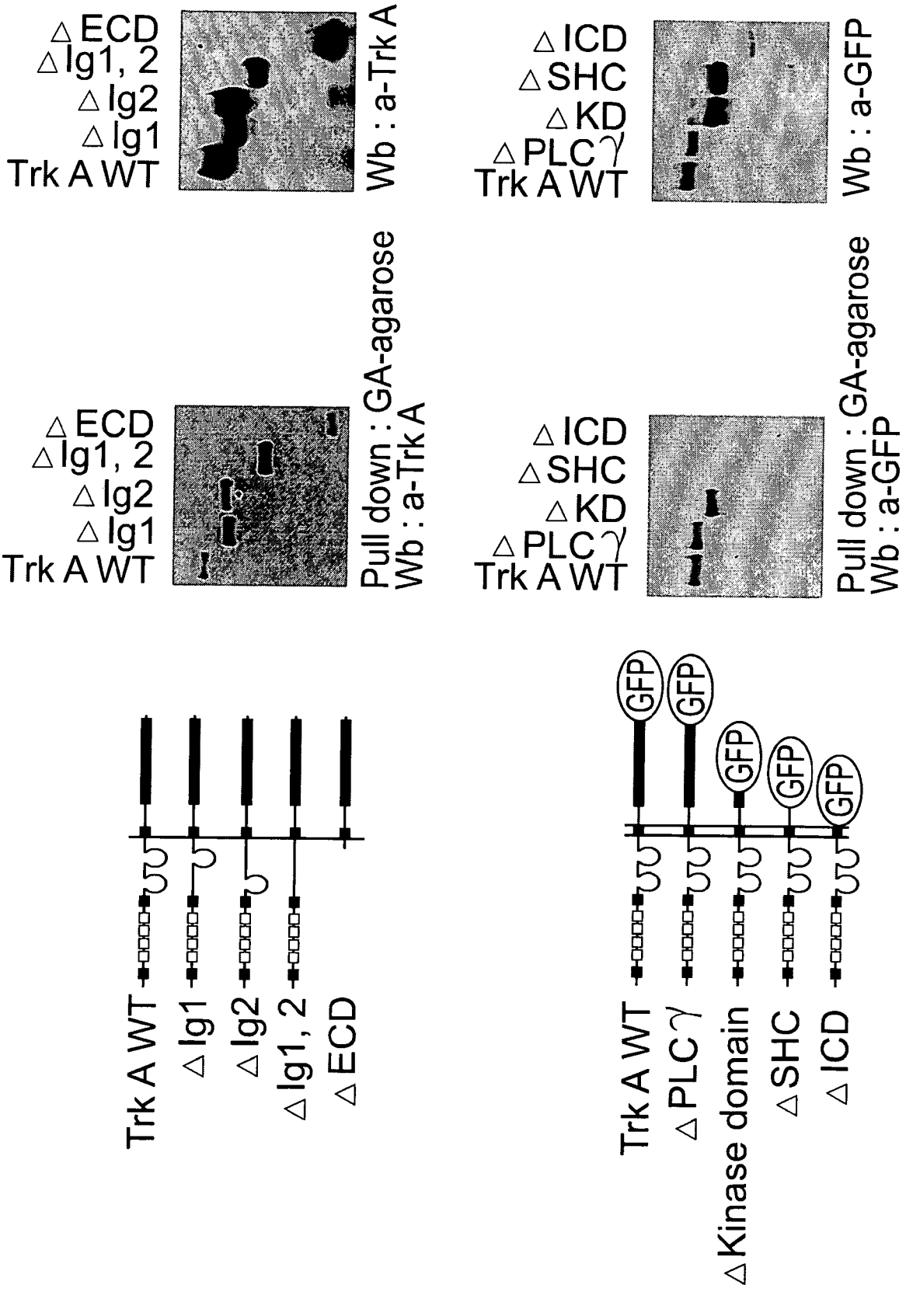

△ECD
△Ig1, 2
△Ig2
△Ig1
Trk A WT
Wb : a-Trk A
Pull down : GA-agarose
Wb : a-Trk A
△ICD
△SHC
△KD
△PLCγ
Trk A WT
Wb : a-GFP
Pull down : GA-agarose
Wb : a-GFP
Trk A WT
△Ig1
△Ig2
△Ig1, 2
△ECD
GFP
Trk A WT
△PLCγ
△Kinase domain
△SHC
△ICD

FIG.6A

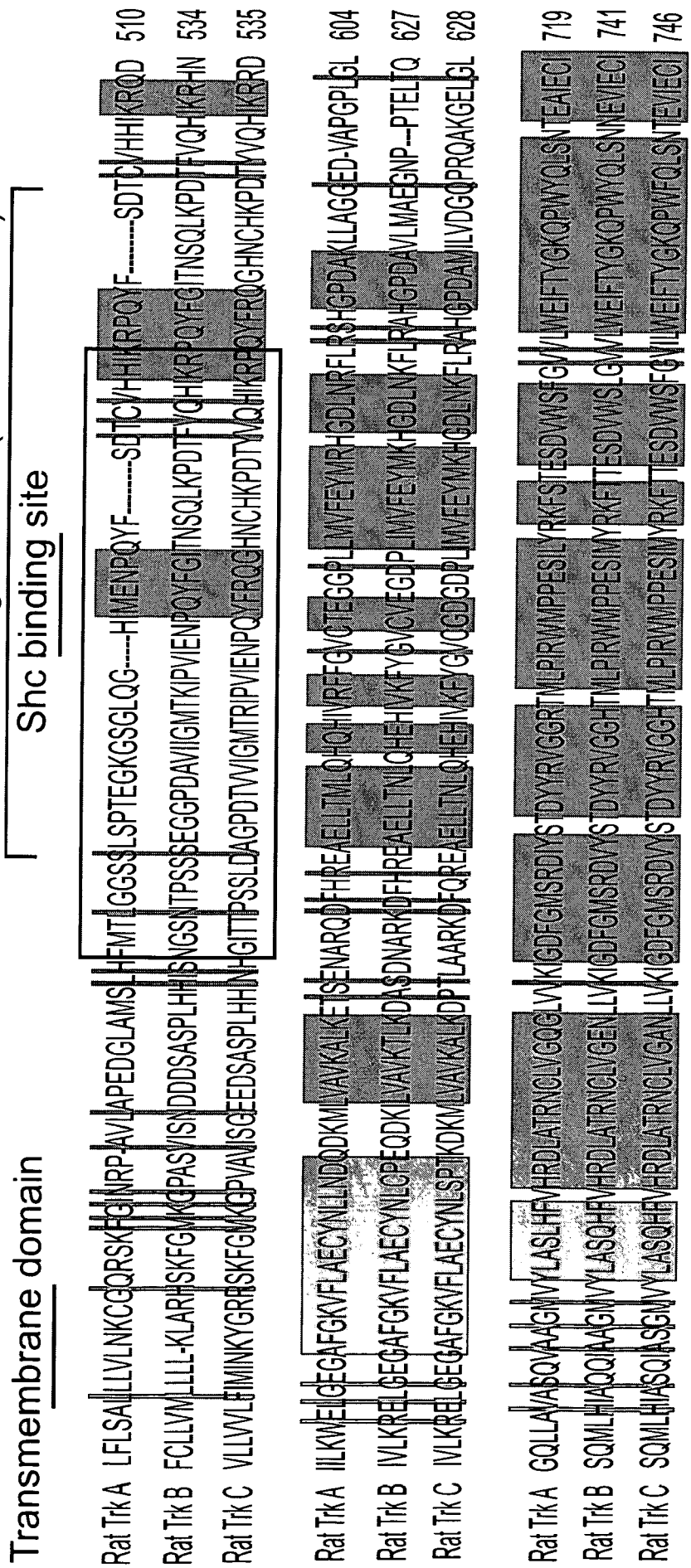

Drug binding domain (a.a. 477-499)
Transmembrane domain
Shc binding site
Rat Trk A
Rat Trk B
Rat Trk C
510
534
535
604
627
628
719
741
746
799
821
825

FIG.6B

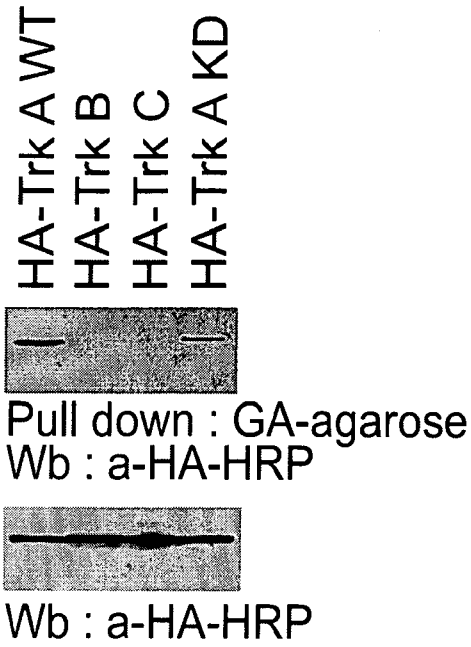



FIG.6C

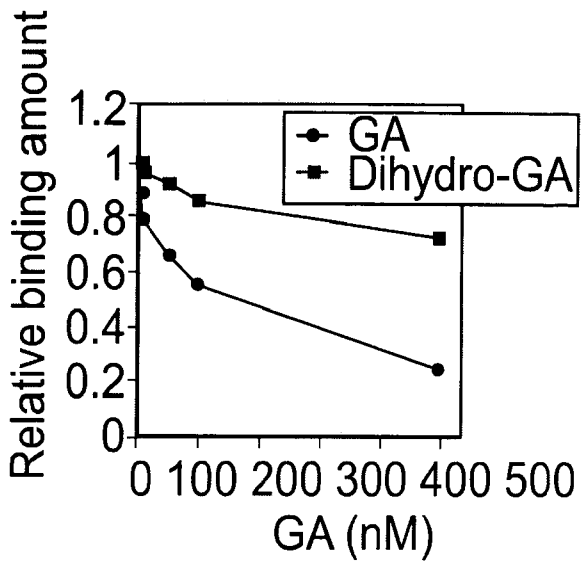



FIG.6D

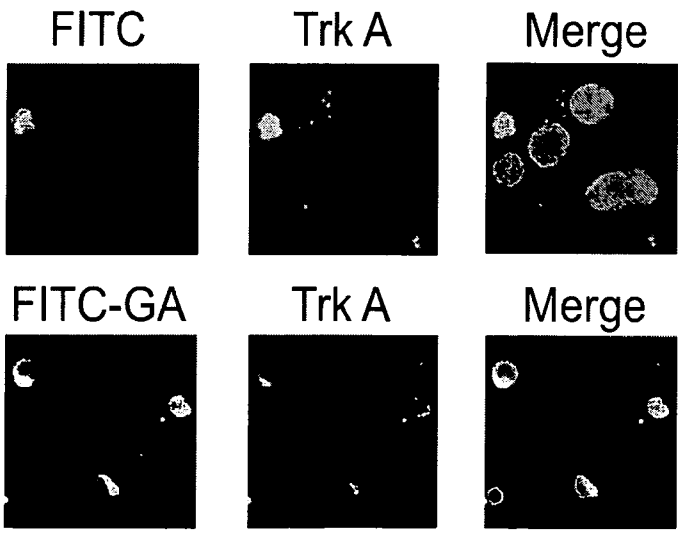
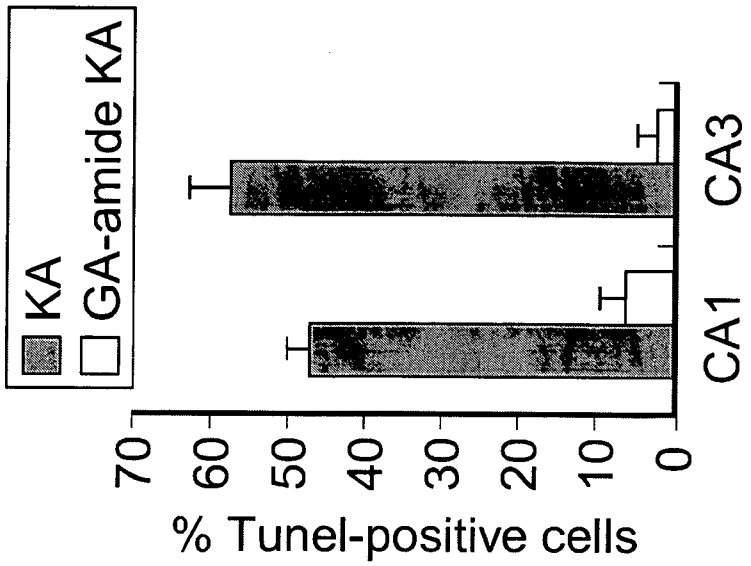



FIG.6E


KA
GA-amide KA
70
60
50
40
30
20
10
0
% Tunel-positive cells
CA1
CA3

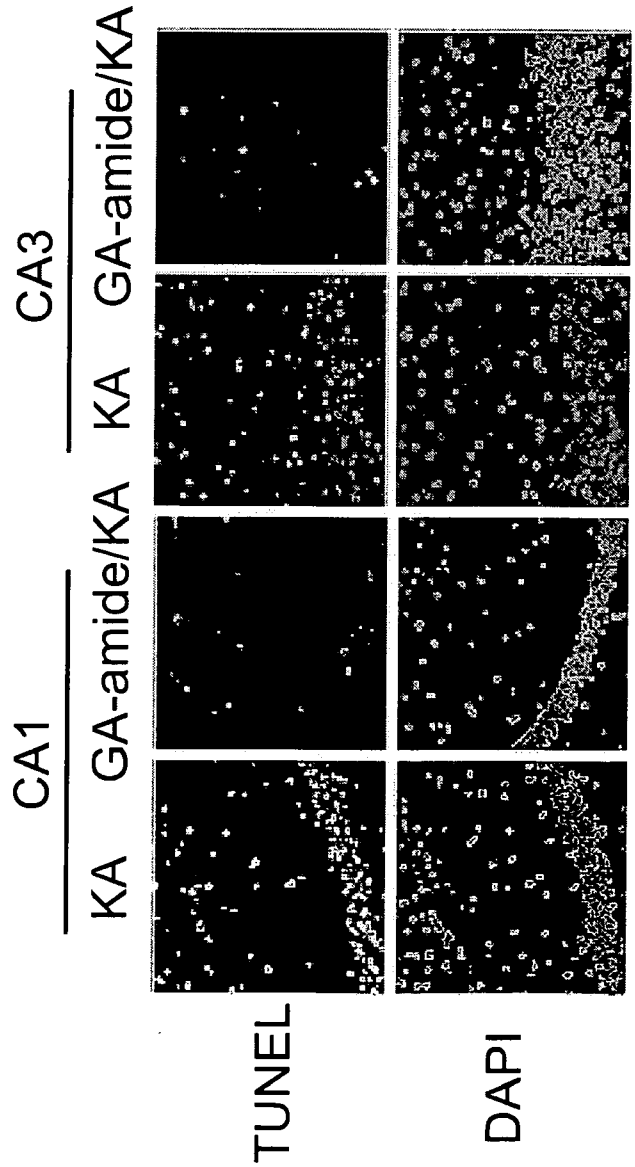
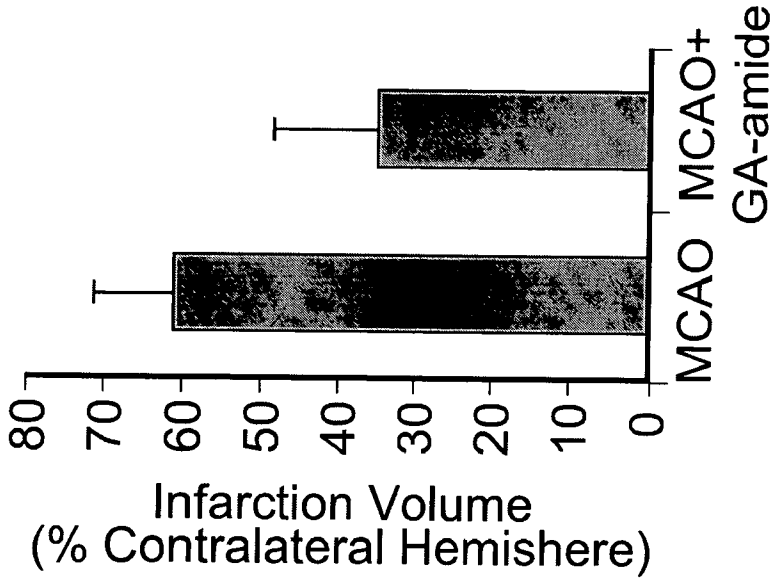

CA1
KA
GA-amide/KA
CA3
KA
GA-amide/KA
TUNEL
DAPI

FIG.7A


80
70
60
50
40
30
20
10
0
Infarction Volume
(% Contralateral Hemishere)
MCAO
MCAO+
GA-amide

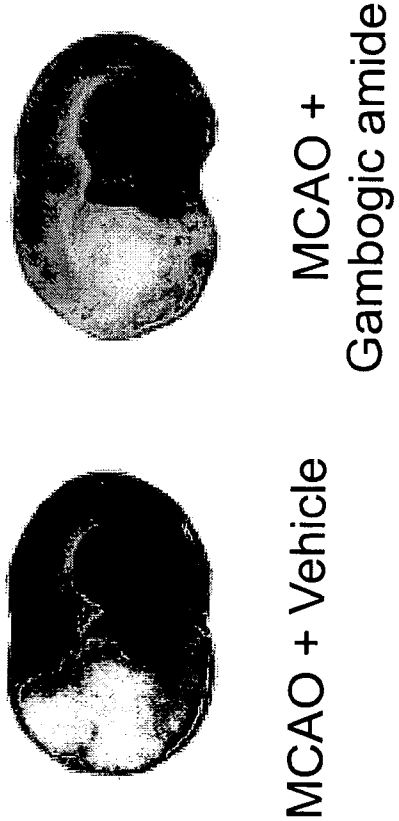

MCAO + Vehicle
MCAO +
Gambogic amide

FIG.7B

**INTERNATIONAL SEARCH REPORT**

International application No.
**PCT/US2008/064723**

**A. CLASSIFICATION OF SUBJECT MATTER**

***A61K 31/352(2006.01)i, A61K 31/45(2006.01)i, A61K 31/497(2006.01)i, C07D 313/06(2006.01)i, C07D 311/78(2006.01)i***

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 8 as above

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKIPASS(KIPO internal), STN(REG, CAplus), PubMed, JPO, USPTO, Google

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X/A | US 2003-0078292 A1 (SUI XIONG CAI, et al) 24 April 2003<br>See the abstract, column [0018]~[0310], examples, claims. | 1-3 / 4, 15-30 |
| X/A | WO 2004-004248 A2 (CYTOVIA, INC.) 8 January 2004<br>See the abstracts, column [0013]~[0101], examples, claims. | 1-3 / 4, 15-30 |
| X/A | WO 2005-060663 A2 (CYTOVIA, INC.) 7 July 2005<br>See the abstract, column [0013]~[0080], examples, claims. | 1-3 / 4, 15-30 |
| A | Wei Liu, et al. Anticancer effect and apoptosis induction of gambogic acid in human gastric cancer line BGC-823. World Journal of Gastroenterology. 2005 Jun 28, 11(24), pp.3655-3659.<br>See the whole documents. | 1-4, 15-30 |
| A | Qing-Long Guo, et al. Inhibition of human telomerase reverse transcriptase gene expression by gambogic acid in human hepatoma SMMC-7721 cells. Life Sciences. 2006 Feb 9, 78(11), pp.1238-1245.<br>See the whole documents. | 1-4, 15-30 |
| A | Andrea la sala, et al. Ligand activation of nerve growth factor receptor TrkA protects monocytes from apoptosis. Journal of Leukocyte Biology. 2000 Jul, 68(1), pp.104-110.<br>See the whole documents. | 1-4, 15-30 |

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 05 NOVEMBER 2008 (05.11.2008) | **05 NOVEMBER 2008 (05.11.2008)** |

Name and mailing address of the ISA/KR
Korean Intellectual Property Office
Government Complex-Daejeon, 139 Seonsa-ro, Seo-gu, Daejeon 302-701, Republic of Korea
Facsimile No. 82-42-472-7140

Authorized officer
Kim, Bum Soo
Telephone No. 82-42-481-5412

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International application No.

**PCT/US2008/064723**

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| US 2003-0078292 A1 | 24.04.2003 | US 6613762 B2 | 02.09.2003 |
| WO 2004-004248 A2 | 08.01.2004 | AU 2003-236960 A1 | 19.01.2004 |
| | | CN 1669280 A | 14.09.2005 |
| | | EP 1527568 A1 | 04.05.2005 |
| | | US 2004-001491 A1 | 01.01.2004 |
| | | US 7023843 B2 | 04.04.2006 |
| | | ZA 200500717 A | 05.08.2005 |
| WO 2005-060663 A2 | 07.07.2005 | US 2007-0093456 A1 | 26.04.2007 |

# INTERNATIONAL SEARCH REPORT

International application No.
**PCT/US2008/064723**

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 5-6, 8-14
because they relate to subject matter not required to be searched by this Authority, namely:

Claims 5-6, 8-14 pertain to methods for treatment of the human or animal body by therapy, as well as diagnostic methods, and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

2. ☒ Claims Nos.: 6, 9-11, 13-14
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claim 6 refers to claim 5, claims 9-11 refer to claim 8, claims 13-14 refer to claim 12, which do not comply with PCT Rule 6.4(a) because multiple dependent claim should not serve as a basis for any other multiple dependent claims. As a result these claims are too unclear to make meaningful search possible.

3. ☒ Claims Nos.: 5, 8, 12
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.