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(54) Title: METHODS AND COMPOSITIONS FOR INHIBITING CELL DEATH

(57) Abstract: The invention relates to products and methods for inhibiting cytochrome c release and/or cell death. The methods of the invention are useful to prevent and treat cytochrome c release-associated diseases and/or disorders such as Alzheimer's disease, Parkinson's disease; Huntington's disease; ALS, epilepsy; stroke, head trauma; spinal cord injury; perinatal hypoxic injury; retinal degeneration; cerebral or other end-organ ischemia inducing cell death associated with a neurosurgical, vascular, cardiac, or transplantation surgery; myocardial infarction; perioperative neuroprotection during carotid endarterectomy; carotid and cardiac stents; abdominal aortic aneurism (AAA) repair; or diabetes.

METHODS AND COMPOSITIONS FOR INHIBITING CELL DEATH

RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. §119(e) of US provisional application 60/789,017, filed April 4, 2006, which is incorporated in its entirety by reference herein.

GOVERNMENT SUPPORT

Aspects of the invention may have been made using funding from the National Institutes of Health/National Institute of Neurological Disorders and Stroke National Institutes of Health Grants 5R01NS039324, 5R01NS041635, and 1R01NS051756-01A1. Accordingly, the Government may have rights in the invention.

FIELD OF THE INVENTION

The invention relates to methods and products for modulating mitochondrial cytochrome c release and for inhibiting cell death. The invention is useful for preventing and treating cell death-associated disorders such as degenerative diseases and disorders, including, but not limited to Alzheimer's disease, Parkinson's disease; Huntington's disease; Amyotrophic Lateral Sclerosis (ALS), epilepsy; stroke, head trauma; spinal cord injury; perinatal hypoxic injury; retinal degeneration; cerebral or other end-organ ischemia inducing cell death associated with a neurosurgical, vascular, cardiac, or transplantation surgery; myocardial infarction; or diabetes, etc. The compositions and methods of the invention can also be used to prevent and/or treat cell death associated with carotid endarterectomy; carotid and cardiac stents; and abdominal aortic aneurism (AAA) repair. The invention also relates in part to assays that are useful for identifying and testing candidate compounds for modulating mitochondrial cytochrome c release.

BACKGROUND OF THE INVENTION

Cell death pathways have emerged as critical and shared components of the pathophysiology of a myriad of neurologic diseases (Friedlander, 2003). The role of the mitochondria in both the regulation of the triggering of a number of cell death pathways and as

key organelles in the generation of energy for the cell places them as pre-eminent targets for therapeutic intervention (Bouchier-Hayes et al., 2005). A common feature in the progression toward cell death is progressive mitochondrial dysfunction with the release of cytochrome c (Beal, 1999; Chan, 2004; Friedlander, 2003; Wang et al., 2003; Zhang et al., 2003b; Zhu et al., 2002). Cytochrome c release from mitochondria is critical and common to neuronal death following various insults to the CNS during acute and chronic neurodegeneration (Friedlander, 2003; Hengartner, 2000; Rigamonti et al., 2001; Wang et al., 2003; Zhang et al., 2003a; Zhu et al., 2002). Following release of cytochrome c in the cytoplasm, an oligomeric complex called the apoptosome is assembled consisting of cytochrome c, pro-caspase-9 and Apaf-1. Assembly of the apoptosome results in caspase-9 activation, which thereafter activates caspase-3 leading to cell death (Li et al., 1997; Zou et al., 1997). Drugs capable of inhibiting mitochondrial release of cytochrome c would be attractive targets for treatment of neurologic diseases featuring caspase-mediated cell death.

SUMMARY OF THE INVENTION

The Food and Drug Administration (FDA) approved drugs have been identified that have unexpected properties of being able to modulate mitochondrial cytochrome c release. The invention relates in part to the use of these newly identified compounds in methods and compositions to prevent and/or treat cell death in neurodegenerative diseases and disorders. We have identified fendiline hydrochoride, calcimycin, dipyrone, ritanserin, azathioprine, bepridil hydrohloride, parthenolide, probucol, methazolamide, hydroxy-progesterone caproate, doxycycline hydrochloride, melatonin, minocycline hydrochloride, mephenytoin, and N-acetyl-DL-tryptophan as compounds that modulate mitochondrial cytochrome c release. Fendiline hydrochoride, calcimycin, dipyrone, ritanserin, azathioprine, bepridil hydrohloride, parthenolide, probucol, methazolamide, hydroxy-progesterone caproate, doxycycline hydrochloride, melatonin, minocycline hydrochloride, mephenytoin, and N-acetyl-DL-tryptophan have not been used in methods to modulate cytochrome c release and the invention includes the use of these compounds to inhibit cytochrome c release and in the treatment of cell death-associated disorders and diseases.

The invention includes, in part, methods for treating disorders resulting from aberrant cytochrome c release and compositions for treating such disorders. In addition we have

identified methods of assaying candidate compounds for the ability to inhibit cytochrome c release.

According to one aspect of the invention, methods for preventing or treating a cell-death associated-disease or disorder in a subject are provided. The methods include administering to a subject in need of such treatment an effective amount of a Group A and/or a Group B cytochrome c release-inhibiting compound to treat the cell death-associated disease or disorder, wherein the subject is otherwise free of indications for treatment with the Group A or Group B cytochrome c release-inhibiting compound that is administered. In some embodiments, a group A cytochrome c release-inhibiting compound is administered. In certain embodiments, the Group A cytochrome c release-inhibiting compound is calcimycin, or an analog, derivative, or variant thereof that inhibits cytochrome c release. In some embodiments, the Group A cytochrome c release-inhibiting compound is dipyrone, or an analog, derivative, or variant thereof that inhibits cytochrome c release. In some embodiments, the group A cytochrome c release-inhibiting compound is parthenolide, or an analog, derivative, or variant thereof that inhibits cytochrome c release. In some embodiments, the Group A cytochrome c release-inhibiting compound is hydroxyprogesterone caproate, or an analog, derivative, or variant thereof that inhibits cytochrome c release. In certain embodiments, the Group A cytochrome c release-inhibiting compound is mephenytoin, or an analog, derivative, or variant thereof that inhibits cytochrome c release. In some embodiments, the Group A cytochrome c release-inhibiting compound is N-acetyl-DL-tryptophan, or an analog, derivative, or variant thereof that inhibits cytochrome c release. In some embodiments, the cell death-associated disease or disorder is Alzheimer's disease, Parkinson's disease; Huntington's disease; Amyotrophic lateral sclerosis (ALS), epilepsy; stroke, head trauma; spinal cord injury; perinatal hypoxic injury; retinal degeneration; cerebral or other end-organ ischemia inducing cell death associated with a neurosurgical, vascular, cardiac, or transplantation surgery; myocardial infarction, or diabetes. In some embodiments, the subject is human. In certain embodiments, the cytochrome c release-inhibiting compound is linked to a targeting molecule. In some embodiments, the targeting molecule's target is a neuronal cell. In some embodiments, the cytochrome c release-inhibiting compound is administered prophylactically to a subject at risk of having a cell death-associated disease or disorder. In certain embodiments, the cytochrome c release-inhibiting compound is administered in combination

with an additional drug for treating a cell death-associated disease or disorder. In some embodiments, the methods also include administering a Group B cytochrome c release-inhibiting compound. In some embodiments, the Group B cytochrome c release-inhibiting compound is fendiline hydrochloride, ritanserin, azathioprine, bepridil hydrochloride, probucol, methoazolamide, doxycycline hydrochloride, melatonin, or minocycline hydrochloride, or an analog, derivative, or variant of fendiline hydrochloride, ritanserin, azathioprine, bepridil hydrochloride, probucol, methoazolamide, doxycycline hydrochloride, melatonin, or minocycline hydrochloride that inhibits cytochrome c release.

According to another aspect of the invention, methods of evaluating the effect of candidate pharmacological agents on cytochrome c release are provided. The methods include contacting a cell-free sample comprising mitochondria with a candidate pharmacological agent; determining the effect of the candidate pharmacological agent on the level of cytochrome c release in the cell-free sample relative to the level of cytochrome c release control cell-free sample not contacted with the candidate pharmacological agent, wherein a relative decrease in the level of cytochrome c release in the cell-free sample as compared with the level in the control cell-free sample indicates the inhibition of cytochrome c release by the candidate pharmacological agent. In certain embodiments, the cell-free sample is contacted with calcium in an amount effective to include cytochrome c release in a control sample. In some embodiments, the amount of cytochrome c release is determined using an ELISA assay. In some embodiments, a relative decrease in the level of cytochrome c release in the cell-free sample contacted with the candidate pharmacological agent compared to the level of cytochrome c release in the cell-free sample not contacted with the candidate pharmacological agent indicates the candidate pharmacological agent is a cytochrome c release-inhibiting compound.

According to yet another aspect of the invention, kits for treating a subject using any of the aforementioned aspects of the invention are provided. The kits include a package housing a first container containing at least one dose of a Group A or a Group B cytochrome c release-inhibiting compound, and instructions for using the cytochrome c release-inhibiting compound in the prevention and/or treatment of an cell death associated disease or disorder. In some embodiments, the container contains at least one dose of a Group A cytochrome c release-inhibiting compound. In certain embodiments, the Group A cytochrome c

release-inhibiting compound is calcimycin, or an analog, derivative, or variant thereof that inhibits cytochrome c release. In some embodiments, the Group A cytochrome c release-inhibiting compound is dipyrone, or an analog, derivative, or variant thereof that inhibits cytochrome c release. In some embodiments, the Group A cytochrome c release-inhibiting compound is parthenolide, or an analog, derivative, or variant thereof that inhibits cytochrome c release. In certain embodiments, the Group A cytochrome c release-inhibiting compound is hydroxyprogesterone caproate, or an analog, derivative, or variant thereof that inhibits cytochrome c release. In some embodiments, the Group A cytochrome c release-inhibiting compound is mephenytoin, or an analog, derivative, or variant thereof that inhibits cytochrome c release. In some embodiments, the Group A cytochrome c release-inhibiting compound is N-acetyl-DL-tryptophan, or an analog, derivative, or variant thereof that inhibits cytochrome c release. In some embodiments, the cell death-associated disease or disorder is Alzheimer's disease, Parkinson's disease; Huntington's disease; ALS, epilepsy; stroke, head trauma; spinal cord injury; perinatal hypoxic injury; retinal degeneration; cerebral or other end-organ ischemia inducing cell death associated with a neurosurgical, vascular, cardiac, or transplantation surgery; myocardial infarction; or diabetes. In certain embodiments, the kit also includes a container containing at least one dose of a Group B cytochrome c release-inhibiting compound, wherein the compound is fendiline hydrochloride, ritanserin, azathioprine, bepridil hydrochloride, probucol, methoazolamide, doxycycline hydrochloride, melatonin, or minocycline hydrochloride, or an analog, derivative, or variant of fendiline hydrochloride, ritanserin, azathioprine, bepridil hydrochloride, probucol, methoazolamide, doxycycline hydrochloride, melatonin, or minocycline hydrochloride that inhibits cytochrome c release and instructions for using the compound for prevention and/or treatment of a cell death associated disease or disorder. In some embodiments, the cytochrome c release-inhibiting compound is formulated for delivery to neuronal cells. In some embodiments, the cytochrome c release-inhibiting compound is formulated for sustained release. In certain embodiments, the cytochrome c release-inhibiting compound is lyophilized.

According to another aspect of the invention, compositions are provided. The compositions include a Group A cytochrome c release-inhibiting compound and a Group B cytochrome c release-inhibiting compound. In some embodiments, the Group A cytochrome c release-inhibiting compound is calcimycin, dipyrone, parthenolide, hydroxyprogesterone

caproate, mephenytoin, or N-acetyl-DL-tryptophan, or an analog, derivative, or variant of calcimycin, dipyrone, parthenolide, hydroxyprogesterone caproate, mephenytoin, or N-acetyl-DL-tryptophan that inhibits cytochrome c release. In certain embodiments, the Group B cytochrome c release-inhibiting compound is fendiline hydrochloride, ritanserin, azathioprine, bepridil hydrochloride, probucol, methoazolamide, doxycycline hydrochloride, melatonin, or minocycline hydrochloride, or an analog, derivative, or variant of fendiline hydrochloride, ritanserin, azathioprine, bepridil hydrochloride, probucol, methoazolamide, doxycycline hydrochloride, melatonin, or minocycline hydrochloride that inhibits cytochrome c release. In some embodiments, the cytochrome c release-inhibiting compound is linked to a targeting molecule. In some embodiments, the targeting molecule's target is a neuronal cell. In certain embodiments, the cytochrome c release-inhibiting compound is formulated for implantation, mucosal administration, injection, inhalation, or oral administration.

According to yet another aspect of the invention, therapeutic methods are provided. The methods include administering a Group A cytochrome c release-inhibiting compound to a subject based on a diagnosis of an cell death and/or cytochrome c release-associated disease in the subject, in an amount effective to treat the disease. In some embodiments, the Group A cytochrome c release-inhibiting compound is calcimycin, or an analog, derivative, or variant thereof. In some embodiments the Group A cytochrome c release-inhibiting compound is dipyrone, or an analog, derivative, or variant thereof. In some embodiments, the Group A cytochrome c release-inhibiting compound is parthenolide, or an analog, derivative, or variant thereof. In some embodiments, the Group A cytochrome c release-inhibiting compound is hydroxyprogesterone caproate, or an analog, derivative, or variant thereof. In some embodiments, the Group A cytochrome c release-inhibiting compound is mephenytoin, or an analog, derivative, or variant thereof. In some embodiments, the cytochrome c release-inhibiting compound is N-acetyl-DL-tryptophan, or an analog, derivative, or variant thereof. In some embodiments of the foregoing aspects of the invention, the cell death and/or cytochrome c release-associated disease, disorder, or procedure is Alzheimer's disease, Parkinson's disease; Huntington's disease; ALS, epilepsy; stroke, head trauma; spinal cord injury; perinatal hypoxic injury; retinal degeneration; cerebral or other end-organ ischemia inducing cell death associated with a neurosurgical, vascular, cardiac, or transplantation surgery; myocardial infarction; perioperative neuroprotection during carotid endarterectomy;

carotid and cardiac stents; abdominal aortic aneurism (AAA) repair; or diabetes. In some embodiments of the foregoing aspects of the invention, the subject is human. In some embodiments of the foregoing aspects of the invention the cytochrome c release-inhibiting compound is linked to a targeting molecule. In some embodiments, the targeting molecule's target is a neuronal cell. In some embodiments of the foregoing aspects of the invention the cytochrome c release-inhibiting compound is administered prophylactically to a subject at risk of having a cell death associated disease or disorder and/or a cytochrome c release-associated disease or disorder. In certain embodiments of the foregoing aspects of the invention the mode of administration is selected from the group consisting of: implantation, mucosal administration, injection, inhalation, and oral administration. In some embodiments of the foregoing aspects of the invention the cytochrome c release-inhibiting compound is administered in combination with an additional drug for treating a cell death-associated disease or disorder and/or a cytochrome c release-associated disease or disorder.

In some embodiments of the foregoing aspects of the invention also include administering a cytochrome c release-inhibiting compound selected from the group consisting of Group B cytochrome c release-inhibiting compounds. In some embodiments, the Group B cytochrome c release-inhibiting compound is fendiline hydrochloride, ritanserin, azathioprine, bepridil hydrochloride, probucol, methoazolamide, doxycycline hydrochloride, melatonin, or minocycline hydrochloride, or an analog, derivative, or variant of fendiline hydrochloride, ritanserin, azathioprine, bepridil hydrochloride, probucol, methoazolamide, doxycycline hydrochloride, melatonin, or minocycline hydrochloride that inhibits cytochrome c release.

The use of the foregoing compositions in the preparation of a medicament, particularly a medicament for prevention and/or treatment of a cytochrome c release-associated disease or disorder and/or a cell death-associated disease or disorder.

These and other objects of the invention will be described in further detail in connection with the detailed description of the invention.

BRIEF DESCRIPTIONS OF THE DRAWINGS

Fig. 1 shows two graphs and a list of experimental results indicating that cytochrome c release is differentially affected by various compounds. Fig. 1a shows overall results of how 80 compounds in kit 6 of the NINDS library were scored. The green line marks the level of

cytochrome release upon stimulation with Ca^{2+} ions in the absence of additional pharmacologic agents. Compounds scored in the interval marked by the green line had no effect on cytochrome release; those scored to its left were inhibitory of the process, and those scored to its right are stimulatory. Additional controls were the levels of cytochrome c release when Ca^{2+} was absent (blue line) and when Ca^{2+}-stimulated release was countered by treatment with cyclosporin A (red line). The X-axis, itself a measure of cytochrome c release, was divided into intervals; the Y-axis shows the number of events falling into each such interval. Fig. 1b is a graph showing colored curves that represent distributions of drug potencies for the 13 kits with 80 compounds in each. In each kit, levels of cytochrome c release were normalized relative to the average of those 80 values. The black line is the sum of these 13 distributions. The dotted line is 1.5 standard deviations less than the mean in the grand distribution. The X-axis was divided into intervals, each spanning 1/20th of the unit distance from zero to the normalized mean. The Y-axis reports the number of measurements (out of the set of 80) that fall into the interval in question. Fig. 1c shows the ranking by effectiveness at inhibiting cytochrome c release from purified mitochondria; of the 21 compounds that were selected. All are in clinical usage, each in the indicated capacity. Whenever known, the molecular target was noted.

Fig. 2. shows graphs and tables of results of screens of neuroprotection ability of 21 compounds in cultured cells. Mutant-htt ST14A striatal cells were shifted to non-permissive temperature to 37°C in serum-deprived medium (SDM) with or without different test drugs for 18 h. Relative cell death was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay. The results are the mean± SD of at least six independent experiments (*, $P < 0.05$, **, $P < 0.001$). Test drugs are divided into group I (Fig. 2a), II (Fig. 2b), and III (Fig. 2c) according to their ability to inhibit cell death and IC_{50} range. The calculated IC_{50}, maximum protection, and/or toxicity afforded by each drug in the different groups are listed in the lower panel, respectively.

Fig. 3 shows graphs and digitized microscopic images of neuronal cells demonstrating that Methazolamide and melatonin inhibit neuronal cell death by preventing the dissipation of $\Delta\Psi m$; methazolamide and melatonin do not inhibit mPT. Fig. 3a shows results of PCNs that

were treated with 1 mM H_2O_2 or 500 μM NMDA for 18 h or kept 18 h after 3 h of OGD treatment in the presence or absence of indicated concentration of methazolamide or melatonin or minocycline or 100 μM test drug in OGD treatment. Cell death was evaluated by the LDH assay (*, P < 0.05; **, P < 0.001). The data are for at least three independent experiments. Empty bars indicate cells without cell death inducers and test drugs. Black solid bars indicate cells with cell death inducer alone. Grey bars indicate cells with cell death inducer plus indicated test drug. Figs. 3b and 3c show results of PCNs treated with OGD (Fig. 3b) or striatal cells were shifted at the non-permissive temperature of 37°C in SDM (Fig. 3c) with or without methazolamide or melatonin or gossypol-acetic acid complex for 5 h and 18 h. Living cells were stained directly with 2 μM RH 123. Arrows show the dissipation of $\Delta\Psi m$ (scale bar, 5 μm). Figs. 3d and 3e show that Methazolamide (Fig. 3d) and melatonin (Fig. 3e) did not prevent mPT induction in isolated liver mitochondria. Mitochondria were incubated in buffer (see Examples section). Mitochondrial swelling was initiated by adding Ca^{2+} 50 μM (Fig. 3d) or 40 μM (Fig. 3e); Ca^{2+} 20 μM + tBH 100 μM (Figs. 3d and 3e); PhAsO 30 μM (Figs 3d and 3e); or Ca^{2+}20 μM + diamide 50 μM (Figs. 3d and 3e) and monitoring in a standard spectroscopic assay at 540 nm. Methazolamide and melatonin were respectively applied to mitochondria in a concentration range from 0.01 μM to 2 mM and from 100 nm to 2 mM.

Fig. 4 shows brain section digitized images, graphs, and Western blots indicating results of the characterization of neuroprotection of methazolamide and melatonin in cerebral ischemia *in vivo*. Lesional sizes (Figs. 4a and 4b) and neuro-scores (Fig. 4c) were assessed in a vehicle stroke group or groups of mice treated with methazolamide (20 mg/kg weight) and melatonin (10 mg/kg weight) at indicated times pre- (1 h) or post- (30 min) MCAO (n = 6 or 7 mice per condition, *, $P < 0.05$, **, $P < 0.001$). The brains were either quickly removed and the coronal sections were cut and stained by 2,3,5-triphenyltetrazolium chloride (Fig. 4a is the staining examples) or extracted to obtain cytosolic fraction lysates (Fig. 4d) or brain lysates (Fig. 4e). The protein samples were separated by SDS/PAGE and probed respectively with antibodies to cytochrome c or to caspase-3. The example of Western blotting was selected as no cerebral ischemia, cerebral ischemia, cerebral ischemia with the addition of methazolamide (post-treatment) or melatonin (pre-treatment); the same blot was reprobed with β-actin antibody (Figs. 4d and 4e). Densitometry was performed to quantify each condition (n =3–5

mice per condition in Figs 4d, 4e, *, $P < 0.05$, **, $P < 0.001$). Empty bars indicate samples without cerebral ischemia and test drugs. Black solid bars indicate samples with cerebral ischemia. Blue or green bars indicate samples with cerebral ischemia plus test drug by pre-treatment or post-treatment, respectively.

Fig. 5 shows drawings of the chemical structure of 21 "hit" compounds are shown as groups I, II, and III.

Fig. 6 shows micrographic images of cells, western blots and graphs indicting that methazolamide and melatonin inhibit the release of mitochondrial apoptogenic factors and forestall the activation of caspase-9 and –3. Fig. 6 A shows cell death is induced in mutant-htt striatal cells by shifting them to the nonpermissive temperature of 37°C in SDM. Test cell cultures contain 100 μM methazolamide or melatonin, whereas controls are devoid of these drugs. After 5 hours, the cells are stained with Mitotracker and then fixed and stained with antibodies to cytochrome c. Mitochondrial cytochrome c is demonstrated as having a punctate pattern that colocalizes with Mitotracker. Cells where cytochrome c is released demonstrate a more diffuse and decreased intensity of cytochrome c staining. Methazolamide and melatonin both retain intensity and punctate characteristics of the cytochrome c staining (Bar = 5 μm). Fig. 6b shows results of mutant-htt striatal cells that were treated with 100 μM methazolamide or melatonin (or processed in the absence of a test drug) for 18 hours. Subsequently, they were extracted and the resulting lysate were separated into cytosolic components. The samples, each of which contains 50 μg of protein, were analyzed by Western blot using antibodies to cytochrome *c*, Smac, caspase-9, or caspase-3. β-actin was used as a loading control. This blot is representative of three independent experiments. Figs. 6c and d show results of mutant-htt striatal cells that were treated with 100 μM methazolamide or melatonin for 5 and 18 hours and compared to untreated controls. Cells were lyzed and their complement of caspase-9 and –3 activities were evaluated by a fluorogenic assay. The results are the mean ± SD of three independent experiments (* indicates that $p < 0.05$, ** that it $p < 0.001$). Experimental controls include cells treated with neither a cell death stimulus nor a test drugs (white bars). Others that receive a death stimulus but are again not treated with a test drug (black bars). Besides these controls, there are measurements of cell death in the presence of methazolamide or melatonin either with or without a death stimulus (gray bars).

Fig. 7. shows micrographic images of cells and graphs indicting that methazolamide and melatonin slow the dissipation of $\Delta\Psi_m$, but do not inhibit mPT. In two models of cell death, striatal cells were transferred to SDM and incubated for 5 or 18 hours at the nonpermissive temperature (Fig. 7**a**) and PCNs were subjected to OGD for 18 hours (Fig. 7b). During these pro-apoptotic treatments, the culture medium was supplemented with methazolamide or melatonin (test drugs) on the one hand, or with gossypol-acetic acid complex or no drug whatsoever (negative controls) on the other. The living cells were then stained with 2 μM Rh 123 in order to determine the electrostatic charge of the mitochondria. (Fig. 7c,d) These drugs were also investigated, this time in the *in vitro* system of purified liver mitochondria that had been stimulated with Ca^{++} ions. Neither methazolamide nor melatonin prevented the induction of the mPT as judged by an unchanging degree of mitochondrial swelling. The lack of such an effect upon addition of methazolamide or melatonin to a solution of stimulated mitochondria is illustrated in (fig. 7c) and (Fig. 7d), respectively. The same is true of mitochondria stimulate in several ways, *i.e.* with Ca^{2+} ions; Ca^{2+} and tBH, PhAsO (Fig. 7c,d); or Ca2+ and diamide. The dose-response curves to these stimuli hardly change when methazolamide is present at 0.01 μM to 2 mM and when melatonin is present from 100 nM to 2 mM. In all cases, mitochondrial swelling is monitored by a standard spectroscopic assay using light of 540 nm. (Fig. 7c,d) Liver mitochondria (0.25 mg/ml) were energized with 5 mM glutamate/malate and incubated with 1, 5, 10 and 20 μM of methazolamide (Fig. 7E) or melatonin (Fig. 7F) in buffer containing 250 mM Sucrose, 10 mM HEPES, 2 mM KH_2PO_4. Mitochondria were challenged with bolus additions of 5 μM Ca^{2+} every 2 min until release of sequestered Ca^{2+} occurred. Alameticin (100 μg) was added in the end of each sample. Upper left panel of Fig. 7e and f is $\Delta\Psi_m$, upper right is in the buffer, lower left is NADH level, and lower right is swelling. See methods for additional information.

Fig. 8 shows digitized images of brain sections, blots and histograms demonstrating that methazolamide and melatonin also diminish damage from cerebral ischemia. Lesion size (Fig. 8 a, b) and neuro-scores (c) were determined for saline-injected mice and ones administered methazolamide (20 mg/kg body weight) or melatonin (10 mg/kg body weight). Drugs were administered either one hour before or 30 minutes after MCAO. Each test and control groups

consisted of 8 mice (n = 8). In all graphs, statistically significant effects are marked with * if $p < 0.05$, and with ** if $p < 0.001$. Brains were quickly removed, cut into coronal sections, and stained with 2,3,5-triphenyltetrazolium chloride (Fig. 8a). In addition, lysates of brain tissue were resolved into cytosolic fractions for analysis by western blotting (Fig. 8 d,e). The protein samples were resolved on SDS/PAGE gels, transferred to nitrocellulose, probed with antibodies to cytochrome c or caspase-3, and reprobed with anti-β-actin. Western blots revealed changes in the cellular localization and degree of maturation of the respective target proteins. (Fig. 8 d,e). Densitometric scans of these gels allowed the signals from cytoplasmic cytochrome c and activated caspase-3 to be compared to that from β-actin. Each test group consisted of 3–5 mice. Again, * indicates that $p < 0.05$ and ** that $p < 0.001$. White bars correspond to brain samples from animals that neither underwent MCAO nor received any test drug. Black bars correspond to samples from saline-injected animals that did undergo MCAO. Blue and green bars correspond to samples from test animals, *i.e.*, one that were both treated with an experimental drug and underwent MCAO.

Fig. 9 shows histograms and a graph demonstration the neuroprotection by dipyrone of cells subjected to OGD and of animals that underwent MCAO. (Fig. 9 a,b) PCNs were incubated for 18 hours with the indicated concentrations of dipyrone starting an hour before a 3 hour of OGD. Control cells were treated identically but in the absence of dipyrone. Cell death was evaluated by the LDH assay (Fig. 9 a). A semi-logarithmic plot of the extent of cell death vs. drug concentration was fitted to a sigmoidal curve. The IC_{50} and maximum protection were then calculated using a computer algorithm (Fig. 9b). White bars correspond to untreated cells that did not undergo OGD. Black bars correspond to untreated cells that underwent 3 hours of OGD. Grey bars corresponding dipyrone-treated cell that underwent 3 hours of OGD. (Fig. 9c,d) Lesion size (Fig. 9c) and neuro-scores (Fig. 9d) were assessed 24 hours after cerebral ischemia. Test mice were injected with dipyrone (10 mg/kg) or the saline vehicle one hour before the start of MCAO (n = 8 mice per test group), * indicates $p < 0.05$ and ** $p < 0.001$.

Fig. 10 shows graphs, a histogram, and digitized images of brain sections demonstrating that methazolamide delays the onset of neurodegeneration in R6/2 mice. (Fig. 10 a, b) Motor performance of R6/2 mice was evaluated by recording the time (up to 7 min) that they remained on a rotarod turning at 15 rpm (Fig. 10a) and 5 rpm (Fig. 10b). Mice were injected daily with

20 mg/kg or 40mg/kg methazolamide (or the saline vehicle) from 6 weeks of age. Measurements were made weekly beginning when the animals were 6 weeks old. p value is compared with saline-treated mice, for 20 mg/kg methazolamide, $p < 0.05$ in weeks 23, 25 and 27; for 40 mg/kg methazolamide, $p < 0.05$ in week 22, 23, 25 and 26, $p < 0.001$ in week 24, 27, and 28. (Fig. 10c) The body weight of the above R6/2 mice was recorded on a weekly basis. (Fig. 10d) The age (in days) at disease onset and at death is tabulated for both drug-treated and control animals. p value is indicated as compared with saline-treated mice (n = 12 for the 20 mg/kg methazolamide-treated group; n = 7 for the 40 mg/kg methazolamide-treated group; n = 18 for saline-injected controls). (Fig. 10e) The brain weight loss in the methazolamide-treated (40 mg/kg) was significantly improved, as compared to the saline-treated R6/2 mice. (Fig. 10f) Both the gross brain atrophy, ventricular enlargement (left), and neuronal atrophy (right) present in saline-treated R6/2 mice was significantly reduced by methazolamide administration. Bar for gross brain equals 2 mm, bar for striatal neurons figures equals 100 μm. Neuropathological data (Fig. 10e, f) were compared by ANOVA or repeated measures of ANOVA and by non-paired Student's t-test (Statview, CA). * indicates $p < 0.05$ and ** $p < 0.001$.

Fig. 11 shows a table demonstrating results of Huntington's mice treated with melatonin at 30 mg/dosage versus saline-treated control mice.

Fig. 12 shows a table demonstrating results of Huntington's mice treated with melatonin at 10 mg/kg dosage versus saline-treated control mice.

Fig. 13 shows graphs demonstrating the results in mice treated with melatonin. Fig. 13A shows results of mice on the rotarod at 15 rpm. Fig. 13B shows results of mice on the rotarod at 5 rpm. Fig. 13C shows body weight comparison between saline control animals and mice treated with melatonin.

DETAILED DESCRIPTION OF THE INVENTION

The methods of the invention involve the administration of compounds that modulate the release of cytochrome c and/or cell death in neuronal and other tissues. Compositions of the

invention include compounds that inhibit the level of cytochrome c release and/or cell death in cells, tissues, and subjects. As used herein, the term "cytochrome c release-inhibiting compounds" means compounds that reduce the level of release of cytochrome c from mitochondria in cells, tissues, and subjects. The methods of the invention involve the administration of cytochrome c release-inhibiting and therefore are useful to reduce or prevent cell death in various degenerative diseases and disorders. As used herein, the terms "cell death-associated disorder" and "cytochrome c release-associated disorder" include, but are not limited to: Alzheimer's disease, Parkinson's disease; Huntington's disease; ALS, epilepsy; stroke, head trauma; spinal cord injury; perinatal hypoxic injury; retinal degeneration; cerebral or other end-organ ischemia inducing cell death associated with a neurosurgical, vascular, cardiac, or transplantation surgery; myocardial infarction; or diabetes. It will be understood that a cell death-associated disorder may result from a medical procedures and/or devices. The methods and compositions of the invention may also be used to prevent or cell death-associated disorders that occur as a result of medical procedures (e.g. surgery, stents, etc.). Examples of types of procedures that may result in cell death treatable by the methods and compositions of the invention, include, but are not limited to, end-organ ischemia associated with a neurosurgical, vascular, cardiac, or transplantation surgery; carotid endarterectomy; carotid and cardiac stents; and abdominal aortic aneurism (AAA) repair.

We have discovered that the deleterious effects seen in these diseases and/or disorders that are triggered by cytochrome c release can be ameliorated by the administration of the compositions of the invention. The compositions of the invention include compounds that inhibit the release of cytochrome c in cells and/or tissues, thereby reducing the cell and tissue damage and clinical manifestations of cytochrome c release-associated diseases and/or disorders.

As used herein, the term "subject" means any mammal that may be in need of treatment with a cytochrome c release-inhibiting compound of the invention. Subjects include but are not limited to: humans, non-human primates, cats, dogs, sheep, pigs, horses, cows, rodents such as mice, rats, etc. Table 1 provides examples of cytochrome c release-inhibiting compounds of the invention.

Table 1. Cytochrome c release-inhibiting compounds.

Primary Name of Compound	Clinical Usage
Group A Compounds	
Calcimycin	Antibiotic; Treatment for hyperparathyroidism
Dipyrone	Non-steroidal anti-inflammatory/anti-pyretic agent
Parthenolide	Anti-inflammatory agent
Hydroxyprogesterone caproate	Hormone therapy
Mephenytoin	Anti-convulsant
N-acetyl-DL-tryptophan (also known as acetyltryptophan)	Antidepressant
Group B Compounds	
Fendiline hydrochloride	Non-selective blocker of calcium channels
Ritanserin	Antagonist to serotonin S2 receptor
Azathioprine	Immunosuppressant; Treatment for rheumatism
Bepridil hydrochloride	Anti-anginal agent
Probucol	Depressant of cholesterol levels
Methazolamide	Inhibitor or carbonic anhydrase used in the treatment of glaucoma
Doxycycline hydrochloride	Antibiotic (tetracycline-derivative that crosses the blood brain barrier)
Melatonin	Pineal gland hormone used to adjust biological clock
Minocycline hydrochloride	Antibiotic (tetracycline-derivative that crosses the blood brain barrier)

The compounds calcimycin, dipyrone, parthenolide., hydroxyprogesterone caproate mephenytoin, and N-acetyl-DL-tryptophan, are known to act as agents in treatment of non-cytochrome c release associated diseases and/or disorders. The foregoing compounds have never before been given to patients to reduce or inhibit cytochrome c release in cells and/or tissues, and/or to treat or prevent a cytochrome c release-associated disorder in subjects who are otherwise free of indications for their administration.

Preferably, the calcimycin, dipyrone, parthenolide, hydroxyprogesterone caproate, mephenytoin, N-acetyl-DL-tryptophan, fendiline hydrochloride, ritanserin, azathioprine, bepridil hydrochloride, probucol, methoazolamide, doxycycline hydrochloride, melatonin, and minocycline hydrochloride compounds of the invention are administered to subjects that are free of indications for their previously determined use. By "free of indications for their previously determined use", it is meant that the subject does not have symptoms that call for treatment with one or more of the compounds of the invention for a previously determined use of that compound, other than the indication that exists as a result of this invention. As used herein the term "previously determined use" of a compound means the use of the compound that was previously identified. Thus, the previously determined use is not the use of inhibiting cytochrome c release in cells and/or tissues.

The methods of the invention include administration of a cytochrome c release-inhibiting compound that preferentially targets neuronal cells and/or tissues or other specific tissues types. In addition, the compounds can be specifically targeted to neuronal tissue (e.g. glial cells and/or neuronal cells) or other specific tissue types. The targeting may be done using various delivery methods, including, but not limited to: administration to neuronal tissue or other specific target tissue, the addition of targeting molecules to direct the compounds of the invention to neuronal or other tissues (e.g. glial cells, neuronal cells, liver cells, heart cells, etc.). Additional methods to specifically target molecules and compositions of the invention to brain tissue and/or neuronal tissues or other tissue types are known to those of ordinary skill in the art.

The invention involves, in part, the administration of a compound that inhibits release of cytochrome c in cells, tissues, and/or subjects. As used herein, the term "release" means the release of cytochrome c from mitochondria. It is understood that the release of cytochrome c from mitochondria may occur following various insults to the CNS during acute and chronic neurodegeneration. The release of cytochrome c from mitochondria of other tissues (e.g. liver, cardiac, vascular, etc) also may result following injury or insult. Release of cytochrome c may result in assembly of the apoptosome, which results in caspase-9 activation, which thereafter activates caspase-3 leading to cell death. The release of cytochrome c from mitochondria is associated with cell death-associated diseases and disorders.

As used herein the term "inhibit" means to reduce the amount of cytochrome c release to a level or amount that is statistically significantly less than a control level of cytochrome c release. In some cases, the decrease in the level of cytochrome c release means the level of cytochrome c release is reduced from an initial level to a level significantly lower than the initial level. In some cases this reduced level may be zero.

A control level of cytochrome c release is the level that represents the normal level of cytochrome c release in a cell, tissue, and/or subject. For example, a control level may be a level that is not associated with cell death. In some instances, a control level will be the level in a disorder-free cell, tissue, or subject, and may be useful, for example, to monitor an increase in the level of cytochrome c release in a cell. In other instances a control level of cytochrome c release will be the level in a cell, tissue, or subject with a neurological disorder, e.g. Alzheimer's disease, or ischemia, and may be useful, for example, to monitor a decrease in the level of cytochrome c release in a cell. These, and other, types of control levels are useful in assays to assess the efficacy of a cytochrome c release-inhibiting compound of the invention.

It will be understood by one of ordinary skill in the art that a control level of cytochrome c release may be a predetermined value, which can take a variety of forms. It can be a single value, such as a median or mean. It can be established based upon comparative groups, such as in disease-free groups that have normal levels of cytochrome c release. Other comparative groups may be groups of subjects with specific neurological disorders, e.g. Alzheimer's disease, Parkinson's disease; Huntington's disease; ALS, epilepsy; stroke, head trauma; spinal cord injury; perinatal hypoxic injury; retinal degeneration; cerebral or other end-organ ischemia inducing cell death associated with a neurosurgical, vascular, cardiac, or transplantation surgery; myocardial infarction; perioperative neuroprotection during carotid endarterectomy; carotid and cardiac stents; abdominal aortic aneurism (AAA) repair; or diabetes. It will be understood that disease-free cells and/or tissues may be used as comparative groups for cells or tissues that have a cytochrome c release-associated disorder.

In some embodiments, a compound that inhibits and thereby reduces the level of cytochrome c released is an agent that reduces a cell death-associated disease or disorder. The level of cytochrome c release may be one that is below the level seen in subjects with a neurological disorder, e.g. may be a level that is clinically asymptomatic.

The invention relates in part to the administration of a cytochrome c release-inhibiting compound of the invention in an amount effective to treat or prevent cytochrome c release in cells, tissues, and/or subjects with a cytochrome c release-associated disease or disorder.

In some aspects of the invention, the cytochrome c release-inhibiting compounds include functional analogs, derivatives, and/or variants of the cytochrome c release-inhibiting compounds of the invention. Thus, the term-cytochrome c release-inhibiting compounds may include functional analogs, derivatives, and/or variants of the calcimycin, dipyrone, parthenolide, hydroxyprogesterone caproate, mephenytoin, N-acetyl-DL-tryptophan, fendiline hydrochloride, ritanserin, azathioprine, bepridil hydrochloride, probucol, methoazolamide, doxycycline hydrochloride, melatonin, and/or minocycline hydrochloride compounds set forth herein. For example, functional analogs, derivatives, and variants of the cytochrome c release-inhibiting compounds of Table 1 may be made to enhance a property of a compound, such as stability. Functional analogs, derivatives, and variants of the compounds of Table 1 may also be made to provide a novel activity or property to a compound of Table 1, for example, to enhance detection, to enhance potency, to reduce side effects, etc. In some embodiments of the invention, modifications to a cytochrome c release-inhibiting compound of the invention, can be made to the structure or side groups of the compound and can include deletions, substitutions, and additions of atoms, or side groups. Alternatively, modifications can be made by addition of a linker molecule, addition of a detectable moiety, such as biotin or a fluorophore, chromophore, enzymatic, and/or radioactive label, and the like.

Analogs of the calcimycin, dipyrone, parthenolide, hydroxyprogesterone caproate, mephenytoin, N-acetyl-DL-tryptophan, fendiline hydrochloride, ritanserin, azathioprine, bepridil hydrochloride, probucol, methoazolamide, doxycycline hydrochloride, melatonin, and/or minocycline hydrochloride compounds that retain some or all of the cytochrome c release-inhibiting activity of the calcimycin, dipyrone, parthenolide, hydroxyprogesterone caproate, mephenytoin, N-acetyl-DL-tryptophan, fendiline hydrochloride, ritanserin, azathioprine, bepridil hydrochloride, probucol, methoazolamide, doxycycline hydrochloride, melatonin, and/or minocycline hydrochloride compounds, respectively, also can be used in accordance with the invention. In some embodiments, an analog of a molecule may have a higher level of cytochrome c release-inhibition activity than the original compound. Chemical groups that can be added to or substituted in the molecules include: hydrido, alkyl, alkenyl,

alkynyl, cycloalkyl, heterocycyl, aryl, heteroaryl, alkoxy, aryloxy, sulfoxy, acyl, amino, acyloxy, acylamino, carboalkoxy, carboxyamido, carboxyamido, halo and thio groups. Substitutions can replace one or more chemical groups or atoms on the molecules.

Molecular terms, when used in this application, have their common meaning unless otherwise specified. The term "hydrido" denotes a single hydrogen atom (H). The term "acyl" is defined as a carbonyl radical attached to an alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl or heteroaryl group, examples of such radicals being acetyl and benzoyl. The term "amino" denotes a nitrogen radical containing two substituents independently selected from the group consisting of hydrido, alkyl, cycloalkyl, heterocyclyl, aryl, and heteroaryl. The term "acyloxy" denotes an oxygen radical adjacent to an acyl group. The term "acylamino" denotes a nitrogen radical adjacent to an acyl group. The term "carboalkoxy" is defined as a carbonyl radical adjacent to an alkoxy or aryloxy group. The term "carboxyamido" denotes a carbonyl radical adjacent to an amino group. The term "carboxy" embraces a carbonyl radical adjacent to an alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl or heteroaryl group. The term "halo" is defined as a bromo, chloro, fluoro or iodo radical. The term "thio" denotes a radical containing a substituent group independently selected from hydrido, alkyl, cycloalkyl, heterocyclyl, aryl and heteroaryl, attached to a divalent sulfur atom, such as, methylthio and phenylthio.

The term "alkyl" is defined as a linear or branched, saturated radical having one to about ten carbon atoms unless otherwise specified. Preferred alkyl radicals are "lower alkyl" radicals having one to about five carbon atoms. One or more hydrogen atoms can also be replaced by a substituent group selected from acyl, amino, acylamino, acyloxy, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxy, nitro, thio, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfoxy, and formyl. Examples of alkyl groups include methyl, *tert*-butyl, isopropyl, and methoxymethyl.

The term "alkenyl" embraces linear or branched radicals having two to about twenty carbon atoms, preferably three to about ten carbon atoms, and containing at least one carbon-carbon double bond. One or more hydrogen atoms can also be replaced by a substituent group selected from acyl, amino, acylamino, acyloxy, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxy, nitro, thio, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfoxy, and formyl. Examples of alkenyl groups include ethylenyl or phenyl ethylenyl.

The term "alkynyl" denotes linear or branched radicals having from two to about ten carbon atoms, and containing at least one carbon-carbon triple bond. One or more hydrogen atoms can also be replaced by a substituent group selected from acyl, amino, acylamino, acyloxy, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxy, nitro, thio, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfoxy, and formyl. Examples of alkynyl groups include propynyl.

The term "aryl" denotes aromatic radicals in a single or fused carbocyclic ring system, having from five to twelve ring members. One or more hydrogen atoms may also be replaced by a substituent group selected from acyl, amino, acylamino, acyloxy, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxy, nitro, thio, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfoxy, and formyl. Examples of aryl groups include phenyl, naphthyl, biphenyl, and terphenyl. "Heteroaryl" embraces aromatic radicals which contain one to four hetero atoms selected from oxygen, nitrogen and sulfur in a single or fused heterocyclic ring system, having from five to fifteen ring members. One or more hydrogen atoms may also be replaced by a substituent group selected from acyl, amino, acylamino, acyloxy, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxy, nitro, thio, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfoxy, and formyl. Examples of heteroaryl groups include, pyridinyl, thiazolyl, thiadiazoyl, isoquinolinyl, pyrazolyl, oxazolyl, oxadiazoyl, triazolyl, and pyrrolyl groups.

The term "cycloalkyl" is defined as a saturated or partially unsaturated carbocyclic ring in a single or fused carbocyclic ring system having from three to twelve ring members. One or more hydrogen atoms may also be replaced by a substituent group selected from acyl, amino, acylamino, acyloxy, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxy, nitro, thio, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfoxy, and formyl. Examples of a cycloalkyl group include cyclopropyl, cyclobutyl, cyclohexyl, and cycloheptyl.

The term "heterocyclyl" embraces a saturated or partially unsaturated ring containing zero to four hetero atoms selected from oxygen, nitrogen and sulfur in a single or fused heterocyclic ring system having from three to twelve ring members. One or more hydrogen atoms may also be replaced by a substituent group selected from acyl, amino, acylamino, acyloxy, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxy, nitro, thio, alkyl, alkenyl,

alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfoxy, and formyl. Examples of a heterocyclyl group include morpholinyl, piperidinyl, and pyrrolidinyl. The term "alkoxy" denotes oxy-containing radicals substituted with an alkyl, cycloalkyl or heterocyclyl group. Examples include methoxy, *tert*-butoxy, benzyloxy and cyclohexyloxy. The term "aryloxy" denotes oxy-containing radicals substituted with an aryl or heteroaryl group. Examples include phenoxy. The term "sulfoxy" is defined as a hexavalent sulfur radical bound to two or three substituents selected from the group consisting of oxo, alkyl, cycloalkyl, heterocyclyl, aryl and heteroaryl, wherein at least one of said substituents is oxo.

The cytochrome c release-inhibiting compounds of the invention also include, but are not limited to any pharmaceutically acceptable salts, esters, or salts of an ester of each compound. Examples of salts that may be used, which is not intended to be limiting include: chloride, acetate, hydrochloride, methansulfonate or other salt of a compound of Table 1 or a functional analog, derivative, variant, or fragment of the compound.

Derivatives of the compounds of Table 1 include compounds which, upon administration to a subject in need of such administration, deliver (directly or indirectly) a pharmaceutically active cytochrome c release-inhibiting compound as described herein. An example of pharmaceutically active derivatives of the invention includes, but is not limited to, pro-drugs. A pro-drug is a derivative of a compound that contains an additional moiety that is susceptible to removal in *vivo* yielding the parent molecule as a pharmacologically active agent. An example of a pro-drug is an ester that is cleaved *in vivo* to yield a compound of interest. Pro-drugs of a variety of compounds, and materials and methods for derivatizing the parent compounds to create the pro-drugs, are known to those of ordinary skill in the art and may be adapted to the present invention.

Analogs, variants, and derivatives of the compounds of the invention set forth in Table 1 may be identified using standard methods known to those of ordinary skill in the art. Useful methods involve identification of compounds having similar chemical structure, similar active groups, chemical family relatedness, and other standard characteristics. For the purposes of this invention, the chemical elements are identified in accordance with the Periodic Table of the Elements, CAS version, Handbook of Chemistry and Physics 75th Ed., inside cover, and specific functional groups are defined as described therein. Additionally, general principles of organic chemistry, as well as specific functional moieties and reactivity, are described in

"Organic Chemistry", Thomas Sorrell, University Science Books, Sausalito. 1999, the contents of which are incorporated herein by reference in their entirety.

Using the structures of the compounds disclosed herein, one of ordinary skill in the art is enabled to make predictions of structural and chemical motifs for analogs, variants, and/or derivatives that possess similar functions of the compounds disclosed in Table 1. Using structural motifs as search, evaluation, or design criteria, one of ordinary skill in the art is enabled to identify classes of compounds (functional variants of the cytochrome c release-inhibiting compounds) that possess the inhibitory function of the compounds disclosed herein. These compounds may be synthesized using standard synthetic methods and tested for activity as described herein. Examples of derivatives, analogs, and variants are known to those of skill in the art. For example, US published patent application No. 20050272716 provides examples of derivatives of parthenolide.

The invention also involves methods for determining the functional activity of cytochrome c release-inhibiting compounds described herein. The function or status of a compound as a cytochrome c release-inhibiting compound can be determined according to assays known as described herein. For example, cells can be contacted with a candidate cytochrome c release-inhibiting compound under conditions that produce cytochrome release, and standard procedures can be used to determine whether cytochrome c release activity is modulated (inhibited or enhanced) by the compound and/or whether cell death levels are modulated by the compound. Such methods may also be utilized to determine the status of analogs, variants, and derivatives as inhibitors of cytochrome c release. Although not intended to be limiting, examples of methods with which the ability of a cytochrome c release-inhibiting compound to inhibit cytochrome c release and/or to inhibit cell death can be tested, are *in vitro* and *in vivo* assay systems provided herein in the Examples section.

Using such assays the level of cytochrome c release can be measured in the system both before and after contacting the system with a candidate cytochrome c release-inhibitor compound as an indication of the effect of the compound on the level of cytochrome c release. Secondary screens may further be used to verify the compounds identified as inhibitors of cytochrome c release and/or cell death.

In addition, derivatives, analogs, and variants of cytochrome c release-inhibiting compounds can be tested for their cytochrome c release-inhibition activity and/or cell death

inhibiting activity by using an activity assay (see examples). An example of an assay method, although not intended to be limiting, is contacting a tissue or cell sample with a cytochrome c release-inhibitor compound and determining the compound's inhibitory activity as described herein. Contacting a similar cell or tissue sample with an analog of the cytochrome c release-inhibiting compound, determining its activity, and then comparing the two activity results as a measure of the efficacy of the analog's cytochrome c release-inhibiting activity.

In addition to the *in vitro* assays described above, an *in vivo* assay may be used to determine the functional activity of cytochrome c release-inhibiting compounds described herein. In such assays, animal models of cytochrome c release-associated disease or disorder can be treated with a cytochrome c release-inhibiting compound of the invention. Cytochrome c release-inhibiting activity and/or cell death may be assayed using methods described herein, which may include labeling or imaging methods. Additionally, animals with and without cytochrome c release-inhibiting compound treatment can be examined for behavior and/or survival as an indication of the effectiveness and/or efficacy of the compounds. Behavior may be assessed by examination of symptoms of aberrant cytochrome c release as described herein. As used herein, the term "aberrant" means abnormal. These measurements can then be compared to corresponding measurements in control animals. For example, test and control animals may be examined following administration of a cytochrome c release-inhibiting compound of the invention. In some embodiments, test animals are administered a cytochrome c release-inhibiting compound of the invention and control animals are not. Any resulting change in cytochrome c release and/or cell death can then be determined for each type of animal using known methods in the art as described herein. Such assays may be used to compare levels of cytochrome c release and/or cell death in animals administered the candidate cytochrome c release-inhibiting compound to control levels of cytochrome c release and/or cell death in animals not administered the cytochrome c release-inhibiting compound as an indication that the putative cytochrome c release-inhibiting compound is effective to reduce cytochrome c release and/or cell death. In other embodiments, a candidate cytochrome c release-inhibiting compound may be administered to both a test and control animal and the results on cytochrome c release and/or cell death may be compared as a measure of the efficacy of the compound.

Once one or more cytochrome c release-inhibiting compounds are verified as inhibiting cytochrome c release using art-known assays or assays as described herein (e.g., in Examples), further biochemical and molecular techniques may be used to identify the targets of these compounds and to elucidate the specific roles that these target molecules play in the process of cytochrome c release and/or cell death in associated diseases and/or disorders. An example, though not intended to be limiting, is that the compound(s) may be labeled and contacted with a cell to identify the host cell proteins with which these compounds interact. Such proteins may be purified, e.g., by labeling the compound with an immunoaffinity tag and applying the protein-bound compound to an immunoaffinity column.

A cytochrome c release-inhibiting compound of the invention may be delivered to the cell using standard methods known to those of ordinary skill in the art. Various techniques may be employed for introducing cytochrome c release-inhibiting compounds of the invention to cells, depending on whether the compounds are introduced *in vitro* or *in vivo* in a host.

When administered, the cytochrome c release-inhibiting compounds (also referred to herein as therapeutic compounds and/or pharmaceutical compounds) of the present invention are administered in pharmaceutically acceptable preparations. Such preparations may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents.

The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. The characteristics of the carrier will depend on the route of administration.

The therapeutics of the invention can be administered by any conventional route, including injection or by gradual infusion over time. The administration may, for example, be oral, intravenous, intraperitoneal, intrathecal, intramuscular, intranasal, intracavity, subcutaneous, intradermal, mucosal, transdermal, or transdermal.

The therapeutic compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing the compounds into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the therapeutic agent into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the therapeutic agent, which is preferably isotonic with the blood of the recipient. This aqueous preparation may be formulated according to known methods using those suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example as a solution in 1, 3-butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono or di-glycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables. Carrier formulations suitable for oral, subcutaneous, intravenous, intramuscular, etc. can be found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA.

Compositions suitable for oral administration may be presented as discrete units such as capsules, cachets, tablets, or lozenges, each containing a predetermined amount of the therapeutic agent. Other compositions include suspensions in aqueous liquors or non-aqueous liquids such as a syrup, an elixir, or an emulsion.

In some embodiments of the invention, a cytochrome c release-inhibiting compound of the invention may be delivered in the form of a delivery complex. The delivery complex may deliver the cytochrome c release-inhibiting compound into any cell type, or may be associated with a molecule for targeting a specific cell type. Examples of delivery complexes include a cytochrome c release-inhibiting compound of the invention associated with: a sterol (e.g., cholesterol), a lipid (e.g., a cationic lipid, virosome or liposome), or a target cell specific binding agent (e.g., an antibody, including but not limited to monoclonal antibodies, or a ligand recognized by target cell specific receptor). Some complexes may be sufficiently stable *in vivo* to prevent significant uncoupling prior to internalization by the target cell. However, the complex can be cleavable under appropriate conditions within the cell so that the cytochrome c release-inhibiting compound is released in a functional form.

An example of a targeting method, although not intended to be limiting, is the use of liposomes to deliver a cytochrome c release-inhibiting compound of the invention into a cell. Liposomes may be targeted to a particular tissue, such neuronal cells, (e.g. hippocampal cells,

etc), or other cell type, by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein. Such proteins include proteins or fragments thereof specific for a particular cell type, antibodies for proteins that undergo internalization in cycling, proteins that target intracellular localization and enhance intracellular half life, and the like.

For certain uses, it may be desirable to target the compound to particular cells, for example specific neuronal cells, including specific tissue cell types, e.g. hippocampal cells or other tissue-specific nervous system cells. In some embodiments, it may be desirable to target a cytochrome c release-inhibiting compound to another cell type, including, but not limited to, liver cells, cardiac cells, vascular cells, etc. In such instances, a vehicle (e.g. a liposome) used for delivering a cytochrome c release-inhibiting compound of the invention to a cell type (e.g. a neuronal cell, liver cell, etc.) may have a targeting molecule attached thereto that is an antibody specific for a surface membrane polypeptide of the cell type or may have attached thereto a ligand for a receptor on the cell type. Such a targeting molecule can be bound to or incorporated within the cytochrome c release-inhibiting compound delivery vehicle. Where liposomes are employed to deliver a cytochrome c release-inhibiting compound of the invention, proteins that bind to a surface membrane protein associated with endocytosis may be incorporated into the liposome formulation for targeting and/or to facilitate uptake.

Liposomes are commercially available from Invitrogen, for example, as LIPOFECTIN™ and LIPOFECTACE™, which are formed of cationic lipids such as N-[1-(2,3 dioleyloxy)-propyl]-N, N, N-trimethylammonium chloride (DOTMA) and dimethyl dioctadecylammonium bromide (DDAB). Methods for making liposomes are well known in the art and have been described in many publications.

The invention provides a composition of the above-described agents for use as a medicament, methods for preparing the medicament and methods for the sustained release of the medicament *in vivo*. Delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of the therapeutic agent of the invention, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. They include, but are not limited to, polymer-based systems such as polylactic and polyglycolic acid, poly(lactide-glycolide), copolyoxalates, polyanhydrides, polyesteramides, polyorthoesters, polyhydroxybutyric acid, and polycaprolactone. Microcapsules of the

foregoing polymers containing drugs are described in, for example, U.S. Pat. No. 5,075,109. Nonpolymer systems that are lipids including sterols such as cholesterol, cholesterol esters and fatty acids or neutral fats such as mono-, di- and tri-glycerides; phospholipids; hydrogel release systems; silastic systems; peptide based systems; wax coatings, compressed tablets using conventional binders and excipients, partially fused implants and the like. Specific examples include, but are not limited to: (a) erosional systems in which the polysaccharide is contained in a form within a matrix, found in U.S. Patent Nos. 4,452,775, 4,675,189, and 5,736,152, and (b) diffusional systems in which an active component permeates at a controlled rate from a polymer such as described in U.S. Patent Nos. 3,854,480, 5,133,974 and 5,407,686. In addition, pump-based hardware delivery systems can be used, some of which are adapted for implantation.

In one particular embodiment, the preferred vehicle is a biocompatible microparticle or implant that is suitable for implantation into the mammalian recipient. Exemplary bioerodible implants that are useful in accordance with this method are described in PCT International application no. WO 95/24929, entitled "Polymeric Gene Delivery System". describes a biocompatible, preferably biodegradable polymeric matrix for containing an exogenous gene under the control of an appropriate promoter. The polymeric matrix is used to achieve sustained release of the exogenous gene in the patient. In accordance with the instant invention, the compound(s) of the invention is encapsulated or dispersed within the biocompatible, preferably biodegradable polymeric matrix disclosed in WO 95/24929. The polymeric matrix preferably is in the form of a microparticle such as a microsphere (wherein the compound is dispersed throughout a solid polymeric matrix) or a microcapsule (wherein the compound is stored in the core of a polymeric shell). Other forms of the polymeric matrix for containing the compounds of the invention include films, coatings, gels, implants, and stents. The size and composition of the polymeric matrix device is selected to result in favorable release kinetics in the tissue into which the matrix device is implanted. The size of the polymeric matrix device further is selected according to the method of delivery which is to be used. The polymeric matrix composition can be selected to have both favorable degradation rates and also to be formed of a material which is bioadhesive, to further increase the effectiveness of transfer when the device is administered to a vascular surface. The matrix composition also can be selected not to degrade, but rather, to release by diffusion over an extended period of time.

Both non-biodegradable and biodegradable polymeric matrices can be used to deliver agents and compounds of the invention of the invention to the subject. Biodegradable matrices are preferred. Such polymers may be natural or synthetic polymers. Synthetic polymers are preferred. The polymer is selected based on the period of time over which release is desired, generally in the order of a few hours to a year or longer. Typically, release over a period ranging from between a few hours and three to twelve months is most desirable. The polymer optionally is in the form of a hydrogel that can absorb up to about 90% of its weight in water and further, optionally is cross-linked with multi-valent ions or other polymers.

In general, the agents and/or compounds of the invention are delivered using the bioerodible implant by way of diffusion, or more preferably, by degradation of the polymeric matrix. Exemplary synthetic polymers which can be used to form the biodegradable delivery system include: polyamides, polycarbonates, polyalkylenes, polyalkylene glycols, polyalkylene oxides, polyalkylene terepthalates, polyvinyl alcohols, polyvinyl ethers, polyvinyl esters, polyvinyl halides, polyvinylpyrrolidone, polyglycolides, polysiloxanes, polyurethanes and co-polymers thereof, alkyl cellulose, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, nitro celluloses, polymers of acrylic and methacrylic esters, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, hydroxy-propyl methyl cellulose, hydroxybutyl methyl cellulose, cellulose acetate, cellulose propionate, cellulose acetate butyrate, cellulose acetate phthalate, carboxylethyl cellulose, cellulose triacetate, cellulose sulphate sodium salt, poly(methyl methacrylate), poly(ethyl methacrylate), poly(butylmethacrylate), poly(isobutyl methacrylate), poly(hexylmethacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate), polyethylene, polypropylene, poly(ethylene glycol), poly(ethylene oxide), poly(ethylene terephthalate), poly(vinyl alcohols), polyvinyl acetate, poly vinyl chloride, polystyrene and polyvinylpyrrolidone.

Examples of non-biodegradable polymers include ethylene vinyl acetate, poly(meth)acrylic acid, polyamides, copolymers and mixtures thereof.

Examples of biodegradable polymers include synthetic polymers such as polymers of lactic acid and glycolic acid, polyanhydrides, poly(ortho)esters, polyurethanes, poly(butic acid), poly(valeric acid), and poly(lactide-cocaprolactone), and natural polymers such as alginate and other polysaccharides including dextran and cellulose, collagen, chemical

derivatives thereof (substitutions, additions of chemical groups, for example, alkyl, alkylene, hydroxylations, oxidations, and other modifications routinely made by those skilled in the art), albumin and other hydrophilic proteins, zein and other prolamines and hydrophobic proteins, copolymers and mixtures thereof. In general, these materials degrade either by enzymatic hydrolysis or exposure to water *in vivo*, by surface or bulk erosion.

Bioadhesive polymers of particular interest include bioerodible hydrogels described by H. S. Sawhney, C. P. Pathak and J. A. Hubell in Macromolecules, 1993, 26, 581-587, the teachings of which are incorporated herein by reference, polyhyaluronic acids, casein, gelatin, glutin, polyanhydrides, polyacrylic acid, alginate, chitosan, poly(methyl methacrylates), poly(ethyl methacrylates), poly(butylmethacrylate), poly(isobutyl methacrylate), poly(hexylmethacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), and poly(octadecyl acrylate).

Use of a long-term sustained release implant may be particularly suitable for treatment of subjects with an established neurological disorder or other cell death-associated condition as well as subjects at risk of developing a neurological disorder, or other cell death-associated condition. "Long-term" release, as used herein, means that the implant is constructed and arranged to deliver therapeutic levels of the active ingredient for at least 7 days, and preferably 30-60 days, and most preferably months or years. The implant may be positioned at or near the site of the neurological damage or the area of the brain or nervous system affected by or involved in the neurological disorder. Long-term release implants may also be used in non-neuronal tissues and organs to allow regional administration of a cytochrome a release-inhibiting compound of the invention. Long-term sustained release implants are well known to those of ordinary skill in the art and include some of the release systems described above.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as

those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

The preparations of the invention are administered in effective amounts. An effective amount is that amount of a pharmaceutical preparation that alone, or together with further doses, stimulates the desired response. In the case of preventing or treating a disorder or condition that is associated with aberrant cytochrome c release, the desired response is reducing the onset, stage or progression of the aberrant cytochrome release and/or cytochrome c release-associated cell death. This may involve only slowing the progression of the damage temporarily, although more preferably, it involves halting the progression of the damage permanently. An effective amount for preventing and/or treating aberrant cytochrome c release is that amount that reduces the amount or level of cytochrome c release, when the cell or subject is a cell or subject with a cytochrome c release-associated disease or disorder, with respect to that amount that would occur in the absence of the active compound.

The invention involves, in part, the administration of an effective amount of a cytochrome c release-inhibiting compound of the invention. The cytochrome c release-inhibiting compounds of the invention are administered in effective amounts. Typically effective amounts of a cytochrome c release-inhibiting compound will be determined in clinical trials, establishing an effective dose for a test population versus a control population in a blind study. In some embodiments, an effective amount will be that amount that diminishes or eliminates a cytochrome c release-associated disease or disorder and/or a cell death-associated disease or disorder in a cell, tissue, and/or subject. Thus, an effective amount may be the amount that when administered reduces the amount of cell death from the amount that would occur in the subject or tissue without the administration of the cytochrome c release-inhibiting compound of the invention.

The pharmaceutical compound dosage may be adjusted by the individual physician or veterinarian, particularly in the event of any complication. A therapeutically effective amount typically varies from 0.01 mg/kg to about 1000 mg/kg, preferably from about 0.1 mg/kg to about 200 mg/kg, and most preferably from about 0.2 mg/kg to about 20 mg/kg, in one or more dose administrations daily, for one or more days. It will be recognized by those of skill in the art that some of the cytochrome c release-inhibiting compounds may have detrimental effects

at high amounts. Thus, an effective amount for use in the methods of the invention may be optimized such that the amount administered results in minimal negative side effects and maximum cytochrome c inhibition.

The absolute amount will depend upon a variety of factors, including the material selected for administration, whether the administration is in single or multiple doses, and individual subject parameters including age, physical condition, size, weight, and the stage of the disease or disorder. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation.

The pharmaceutical compounds of the invention may be administered alone, in combination with each other, and/or in combination with other drug therapies that are administered to subjects with cell death-associated diseases and/or disorders. Additional drug therapies (for treatment and/or prophylaxis) that may be administered with pharmaceutical compounds of the invention include, art-known methods of treating cell death-associated diseases and/or disorders and/or cytochrome c release-associated diseases and/or disorders. Alternative drug therapies are known to those of ordinary skill in the art and are administered by modes known to those of skill in the art. The drug therapies are administered in amounts that are effective to achieve the physiological goals (to reduce symptoms and damage from cytochrome c release-associated disease or disorder in a subject, e.g. cell death), in combination with the pharmaceutical compounds of the invention. Thus, it is contemplated that the alternative drug therapies may be administered in amounts which are not capable of preventing or reducing the physiological consequences of the cytochrome c release-associated disease and/or disorder when the drug therapies are administered alone, but which are capable of preventing or reducing the physiological consequences of a cytochrome c release-associated disease and/or disorder when administered in combination with one or more cytochrome c release-inhibiting compounds of the invention.

Diagnostic tests known to those of ordinary skill in the art may be used to assess the level of cytochrome c release and/or the level of cell death in a subject and to evaluate a therapeutically effective amount of a pharmaceutical compound administered. Examples of diagnostic tests are set forth below. A first determination of cytochrome c release in a cell and/or tissue may be obtained using one of the methods described herein (or other methods known in the art), and a second, subsequent determination of the level of cytochrome c release

may be done. A comparison of the cytochrome c release activity and/or the level of cell death at the different time points may be used to assess the effectiveness of administration of a pharmaceutical compound of the invention as a prophylactic or a treatment of the cytochrome c release-associated disease or disorder and/or a cell death-associated disease or disorder. Family history or prior occurrence of a cytochrome c release-associated disease or disorder, even if the cytochrome c release-associated disease or disorder is absent in a subject at present, may be an indication for prophylactic intervention by administering a pharmaceutical compound described herein to reduce or prevent aberrant cytochrome c release.

An example of a method of diagnosis of aberrant cytochrome c release that can be performed using standard methods such as, but not limited to: imaging methods, electrophysiological methods, and histological methods. Additional methods of diagnosis and assessment of cytochrome c release-associated disease or disorders and the resulting cell death or damage are known to those of skill in the art.

In addition to the diagnostic tests described above, clinical features of cytochrome c release-associated diseases and/or disorders can be monitored for assessment of cytochrome c release following onset of a cytochrome c release-associated disease or disorder. These features include, but are not limited to: assessment of the presence of cell death, neuronal cell lesions, spinal cord lesions, brain lesions, organ lesions, and behavioral abnormalities. Such assessment can be done with methods known to one of ordinary skill in the art, such as behavioral testing and imaging studies, such as radiologic studies, CT scans, PET scans, etc.

The invention also provides a pharmaceutical kit comprising one or more containers comprising one or more of the cytochrome c release-inhibiting compounds of the invention and/or formulations of the invention. The kit may also include instructions for the use of the one or more cytochrome c release-inhibiting compounds or formulations of the invention for the treatment of a cytochrome c release-associated disease or disorder. The kits of the invention may also comprise additional drugs for preventing and/or treating a cytochrome c release-associated disease or disorder.

The invention further provides efficient methods of identifying pharmacological agents or lead compounds for agents and compounds that inhibit (or enhance) cytochrome c release. Generally, the screening methods involve assaying for compounds which modulate (enhance or inhibit) the level of cytochrome c release. As will be understood by those of ordinary skill in

the art, the screening methods may measure the level of cytochrome c release directly, e.g., screening methods described herein. In addition, screening methods may be utilized that measure a secondary effect of cytochrome c release, or example, the level of cell death in a cell or tissue sample or by measuring behavioral characteristics of the cytochrome c release-associated disease.

A wide variety of assays for pharmacological agents can be used in accordance with this aspect of the invention, including, cytochrome c release assays, cell viability assays, cell-free assays, cell-based assays, etc. As used herein, the term "pharmacological agent" means cytochrome c release-inhibiting compounds. An example of such an assay that is useful to test candidate cytochrome c release-inhibiting compounds is provided in the Examples section. In such assays, the assay mixture comprises a candidate pharmacological agent. Typically, a plurality of assay mixtures is run in parallel with different agent concentrations to obtain a different response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e., at zero concentration of agent or at a concentration of agent below the limits of assay detection.

Candidate compounds useful in accordance with the invention encompass numerous chemical classes, although typically they are organic compounds. Preferably, the candidate pharmacological agents are small organic compounds, i.e., those having a molecular weight of more than 50 yet less than about 2500, preferably less than about 1000 and, more preferably, less than about 500. Candidate compounds comprise functional chemical groups necessary for structural interactions with proteins and/or nucleic acid molecules. The candidate compounds can comprise cyclic carbon or heterocyclic structure and/or aromatic or polyaromatic structures substituted with one or more of the above-identified functional groups. Candidate compounds also can be biomolecules such as peptides, saccharides, fatty acids, sterols, isoprenoids, purines, pyrimidines, derivatives or structural analogs of the above, or combinations thereof and the like. Where the compound is a nucleic acid molecule, the agent typically is a DNA or RNA molecule, although modified nucleic acid molecules are also contemplated.

It is contemplated that cell-based assays as described herein can be performed using cell samples and/or cultured cells. Biopsy cells and tissues as well as cell lines grown in culture are useful in the methods of the invention.

Candidate compounds are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides, synthetic organic combinatorial libraries, phage display libraries of random peptides, and the like. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural and synthetically produced libraries and compounds can be readily be modified through conventional chemical, physical, and biochemical means. Further, known pharmacological compounds may be subjected to directed or random chemical modifications such as acylation, alkylation, esterification, amidification, etc. to produce structural analogs of the compounds. Candidate compounds also include analogs, derivatives, and/or variants of the cytochrome c release-inhibiting compounds described herein.

A variety of other reagents also can be included in the mixture. These include reagents such as salts, buffers, neutral proteins (e.g., albumin), detergents, etc. which may be used to facilitate optimal binding, or to reduce non-specific or background interactions of the reaction components. Other reagents that improve the efficiency of the assay such as protease inhibitors, nuclease inhibitors, antimicrobial agents, and the like may also be used.

An exemplary cytochrome c release-inhibitor assay is described herein, which may be used to identify candidate compounds that inhibit cytochrome c release and/or cell death. In general, the mixture of the foregoing assay materials is incubated under conditions whereby, but for the presence of the candidate pharmacological compound, there is an above-normal level of cytochrome c release and/or cell death. The order of addition of components, incubation temperature, time of incubation, and other parameters of the assay may be readily determined. Such experimentation merely involves optimization of the assay parameters, not the fundamental composition of the assay. Incubation temperatures typically are between 4°C and 40°C. Incubation times preferably are minimized to facilitate rapid, high throughput screening, and typically are between 0.1 and 10 hours.

After incubation, the level of cytochrome c release may be detected by any convenient method available to the user. In some embodiments, an ELISA assay may be used for detection. Detection may be effected in any convenient way for cell-based assays. For cell-based assays, one of the components usually comprises, or is coupled to, a detectable label.

A wide variety of labels can be used, such as those that provide direct detection (e.g., radioactivity, luminescence, optical or electron density, etc.) or indirect detection (e.g., epitope tag such as the FLAG epitope, enzyme tag such as horse-radish peroxidase, etc.).

A variety of methods may be used to detect the label, depending on the nature of the label and other assay components. Labels may be directly detected through optical or electron density, radioactive emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, strepavidin-biotin conjugates, etc. Methods for detecting the labels are well known in the art.

The invention will be more fully understood by reference to the following examples. These examples, however, are merely intended to illustrate the embodiments of the invention and are not to be construed to limit the scope of the invention.

EXAMPLES

Introduction

Because blocking cytochrome c release from the mitochondria may support a novel therapeutic approach to neurodegeneration, we have developed a rapid assay in purified mitochondria to screen the inhibitors of cytochrome c release. Inhibitors of cytochrome c release were identified from a library of 1,040 drugs approved by the Food and Drug Administration. Effective drugs at the level of purified mitochondria were evaluated in several paradigms of neuronal cell death. Two compounds, methazolamide and melatonin, were evaluated in further depth in cellular and *in vivo* models of neuronal injury. We demonstrated that these compounds prevented neuronal cell death by blocking the dissipation of mitochondrial membrane potential ($\Delta\Psi m$). Furthermore, we demonstrated that methazolamide and melatonin reduced infarct size and decreased neurologic deficit in a mouse model of cerebral ischemia. Our findings demonstrate the utility of a mitochondrial screen for the identification of inhibitors of cell death. Given that these are FDA approved drugs, and many of them effectively cross the blood brain, these drugs may expeditiously progress into human trials of neurologic diseases featuring caspase-mediated cell death.

We previously demonstrated that minocycline, a broadly neuroprotective drug, acts at least in part by directly inhibiting the release of cytochrome c from mitochondria (Shan, 2002).

Minocycline is protective in experimental models of stroke, traumatic brain and spinal cord injury, HD, ALS, Parkinson disease, and multiple sclerosis (Brundula et al., 2002; Chen et al., 2000; Friedlander, 2003; Wang et al., 2003; Wu et al., 2002; Yrjanheikki et al., 1998; Zhu et al., 2002). From the mechanistic insight we generated evaluating minocycline (Wang et al., 2003; Zhu et al., 2002), and with the goal of identifying inhibitors of cytochrome c release, we developed a cell-free screening assay to identify drugs that would inhibit cytochrome c release from purified mitochondria. A Neurodegeneration Drug Screening Consortium was organized by the National Institute of Neurological Disorders and Stroke (NINDS) to screen a library of 1,040 compounds (the library is enriched in drugs approved by the Food and Drug Administration [FDA], and many of them are able to cross the blood-brain barrier).

The drug screen as been performed and results analyzed with respect to the identification of inhibitors of the release of cytochrome c from purified mitochondria. Some of the promising drugs from the cell-free screen were also subjected to secondary screens in neuronal cell lines. Some of the drug hits were thereafter evaluated in primary cerebrocortical neurons. Two drugs were also demonstrated to be protective in an *in vivo* model of cerebral ischemia. Our results suggest, for the first time, that inhibitors of cytochrome c release might be effective as therapeutic agents for neurodegeneration.

Example 1

Methods

Drugs. The drugs were obtained from the 1,040 compounds National Institute of Neurological Disorders and Stroke library by Custom Collection of Microsource Discovery Systems Inc., or were obtained from Sigma-Aldrich (St. Louis, MO).

Cell lines and stimulation of cell death. The cell line ST14A are striatal neurons conditionally immortalized by transfection with a temperature-sensitive form of the SV40 large T-antigen. N548mu [nt1955–128] stable huntingtin ST14A (mutant-htt ST14A) cells were cultured as described (Rigamonti et al., 2000). Mutant-htt ST14A cells were kindly provided by Dr. Elena Cattaneo (University of Milan, Italy). Cells were shifted to the non-permissive temperature of 37°C in serum-deprived medium (SDM) for the indicated time with or without different concentrations of methazolamide, melatonin, minocycline, or gossypol-acetic acid. Culture of

primary cortical neurons (PCN) (E14-E16) was performed as previously described (Zhang et al., 2003b). PCN were stimulated with 1 mM H_2O_2 or 500 μM NMDA for 18–20 h, or OGD as described previously (Zhang et al., 2003b). Briefly, for OGD treatment, the culture medium was replaced with glucose-free Earle's balanced salt solution with or without different concentrations of test drugs, and the cells were placed in an anaerobic chamber with BBL GasPak Plus (Becton Dickinson), which reduces the oxygen concentration to <100 ppm within 90 min. Control cells were incubated in Earle's balanced salt solution with glucose in a normoxic incubator for the same period. After 3 h, OGD was terminated by a return to normal culture conditions. All experiments used 2 h of pre-incubation with test drugs. Cell death was determined by MTS or LDH assay.

MTS assay. The inhibition ability of cell death by individual 21 compounds was screened in mutant-htt ST14A striatal cells. The cell death was determined by the 3-(4,5)-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay. The assay was performed as described previously (Wang et al., 2003) and according to the manufacturer's instructions (Promega).

LDH assay. Primary cortical neuron cell death was determined by the LDH assay according to the manufacturer's instructions (Hoffmann-La Roche Inc. (Roche), Nutley, N.J.,) (Zhang et al., 2003b).

Enzyme-linked immunosorbent assay (ELISA). The NINDS library was obtained as 13 kits with 80 compounds in each. A single 96-well ELISA plate was dedicated to each kit of 80 compounds. Drugs were assayed with a separate mitochondrial preparation for each kit. For each sample within a given 96-well plate, the absorbance at 450 nm (a measure of cytochrome c concentration) was corrected for imperfections in the plastic by subtracting the reading at 540 nm. Cytochrome c concentration was calculated by interpolating these values on a standard curve constructed for each plate.

Determination of $\Delta\Psi m$. Striatal cells and PCNs were treated as indicated with or without methazolamide or melatonin or gossypol-acetic acid complex. Rhodamine 123 (Rh 123)

staining (Molecular Probes, [Invitrogen, Carlsbad, CA]) (2 μM for 5 min) was performed in living cells. Deconvoluted images were taken with a Nikon ECLIPSE TE 200 fluorescence microscope and processed with IP LAB software (Spectra Services, Webster, NY).

Measurement of mitochondrial permeability transition (mPT). Rat liver mitochondria were isolated from 4-month-old male Fisher 344 × Brown Norway F_1 rats by differential centrifugation, as described previously (Wang et al., 2003; Zhu et al., 2002). The isolation buffer contained 0.3 M sucrose, 10 mM HEPES, 1 mM EGTA, and 0.5% BSA. The protein content was determined by the Lowry method with bovine serum albumin as a standard. Isolated mitochondria were then incubated in the buffer containing 250 mM sucrose, 10 mM HEPES, pH 7.4, 2.5 mM KH_2PO_4, and 5 mM K^+-succinate or glutamate/malate, mitochondria 1 mg/ml. Mitochondria swelling was induced by Ca^{2+}, tert-butylhydroperoxide (tBH), PhAsO, or diamide and monitored in standard spectroscopic assay at 540 nm. Methazolamide and melatonin were respectively applied to mitochondria.

Mice and Treatment

Permanent middle cerebral artery occlusion (MCAO). Focal cerebral ischemia was produced as described previously (Zhang et al., 2003b). Briefly, anesthesia was induced in 6- to 8-week-old C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) with 2% (vol/vol) isoflurane (70% N_2O/30% O_2) and was maintained with a 1-0.5% concentration. Rectal temperature was maintained between 37.0 and 37.5°C with a heating pad (Harvard Apparatus, Hamden, CT). Focal cerebral ischemia was induced by an intraluminal 7-0 nylon thread with a silicone tip (180 μm diameter) introduced into the right cervical internal carotid artery. The thread was inserted 9 ± 1.0 mm into the internal carotid artery up to the middle cerebral artery. A laser Doppler perfusion monitor (Perimed AB, Järfälla, Sweden) was adhered to the right temporal aspect of the animal's skull and used to confirm middle cerebral artery flow disruption. Control animals underwent sham surgery consisting of only anesthesia and carotid artery dissection. All animals were sacrificed after surgery. The animal's brain was removed, and the cerebral hemisphere was retained for either evaluation of infract volume and neuroscore or Western blot analysis.

Determination of Infarct Volume

After 24 h of MCAO, the mice were killed. The brains were rapidly removed and chilled for 2 min. Coronal sections (1-mm thick; $n = 7$) were cut with a mouse brain matrix. Each slice was immersed in a saline solution containing 2% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich) at 37°C for 20 min. After staining, each slice was scanned with an HP scanjet 4200C. The stained and unstained areas of the right hemisphere were quantified with imageJ 1.32j (NIH), and these values were used to calculate the volume infarct expressed as a percentage of the lesioned hemisphere.

Neurobehavioral Examination

Mice were assigned a neurobehavioral score at 2 and 24 h following MCAO. A previously reported scale was used (Stavrovskaya et al., 2004): 0, no neurological deficits; 1, failure to extend the left forepaw; 2, circling to contralateral side; 3, loss of ability to walk or righting reflex; and 4, death before 24 h.

Western blot. Samples of mouse brain were lysed in RIPA buffer with protease inhibitors (Wang et al., 2003; Zhu et al., 2002). Antibody to caspase-3 were purchased from Cell Signaling Technology (Beverly, MA), antibody to β-actin was from Sigma, and secondary antibodies and ECL reagents were from Amersham Pharmacia Biotech (GE Healthcare Life Sciences, Piscataway, NJ).

Fractionation of cell and tissue. Mouse brain cytosolic fractionations were performed as described (Wang et al., 2003; Zhang et al., 2003b; Zhu et al., 2002). Released cytochrome c was analyzed by Western blot. Antibody to cytochrome c was purchased from PharMingen (San Diego, CA).

Statistical analysis. Densitometric quantification was performed with the Quantity One Program (Bio-Rad). Statistical significance was evaluated by t-test: p values < 0.05 were considered significant and are indicated (*), and ** indicates p values < 0.001. Drug analysis, including IC_{50} and maximum protection, were performed by GraphPad Prism program (GraphPad Software, San Diego, CA).

Results

Library screen for inhibitors of cytochrome c release from purified mitochondria.

To search for inhibitors of cytochrome c release, we developed a cell-free screening assay to identify drugs that would inhibit cytochrome c release from purified mouse liver mitochondria. Mitochondria were preincubated with the drug. To induce cytochrome c release, calcium was then added. Following a 30 minute incubation at 37°C, the mitochondria were pelleted. Using an ELISA, we quantified cytochrome c released from the mitochondria. We screened a library of 1,040 compounds for the ability to inhibit the release of cytochrome c from purified mitochondria of rat liver. The 1,040 compounds assembled by the Neurodegeneration Drug Screening Consortium of the NINDS provide a wealth of potentially neuroprotective agents. Most of them are FDA-approved drugs in current clinical usage (Heemskerk et al., 2002).

The ELISA was performed by a double-blind method. We were not aware of the identity of the specific drugs until we provided the results to the NINDS data repository. Fig. 1a is a representative graph generated from one of the 13 kits. The data for each set of 80 compounds is ultimately represented as a distribution of their impact upon cytochrome c release. Most drugs have little effect on the release of cytochrome c in response to Ca^{2+} stimulation. However, there are outlying points, indicative of drugs that substantially stimulate or, more significantly, inhibit cytochrome c release. In Fig. 1b, the data for the entire NINDS library are plotted slightly differently: cytochrome c release for each compound is normalized with respect to the average value for the respective kit (these averages differ among the 13 kits, although the mean values for groups of 80 statistics should not vary by much). This algorithm generates the colored curves from the data on the 13 kits comprising the NINDS library. The distribution of a sum of all 13 of these curves, is represented by the solid black line.

To choose potential effective inhibitors of cytochrome c release, we selected drugs that inhibited release with a potency 1.5 standard deviations to the left of the mean (dotted line). In the primary screen, 48 compounds inhibited cytochrome c release below this value. Upon a secondary and confirmatory screen, 39 of these top candidates were confirmed to be effective at concentrations of 10 and 20 μM. We then selected among them on the basis of their known ability to cross the blood-brain barrier and their current usage (to treat other conditions) in

humans. We rejected drugs with significant side effects and an inability to cross the blood-brain barrier, leaving 21 compounds (Fig. 1c). Thus, our cell-free assay identified just under 2% of the 1,040 compounds holding the greatest promise for use as neuroprotective agents.

Screen of cultured cells provides a class of inhibitors of cytochrome c release as potential drugs for neurodegeneration.

The response of whole living cultured cells to pharmacological agents is more complex than that of isolated mitochondria. Consequently, the most potent agents in purified organelles will not necessarily be the best drugs in cell culture. We therefore further screened the ability of the 21 compounds that blocked cytochrome c release from purified mitochondria to inhibit cell death in mutant-htt ST14A cell lines, a well established cellular model of HD (Rigamonti et al., 2000; Wang et al., 2005; Wang et al., 2003), in which the release and inhibition of cytochrome c play an important role in neurodegenerative disease (Rigamonti et al., 2001; Wang et al., 2003). As previously reported (Rigamonti et al., 2000; Wang et al., 2005; Wang et al., 2003) shifting temperature-sensitive mutant-htt ST14A cells from 33°C (permissive temperature) to 37°C (non-permissive temperature) in serum-deprived medium causes the death of mutant-htt ST14A cells.

We measured the ability of each drug to inhibit cell death, and graphed the extent of cell death against the drug concentration (on a logarithmic scale), which provided the curves and the IC_{50} and maximum protection values (Fig. 2). Although the dose-response curves of the tested drugs vary, they fall into three broad categories. Group I and II drugs significantly protect viability as cells shift to the non-permissive temperature. Group I compounds are active at lower concentrations (with the IC_{50} in the nanomolar range) but are toxic at high levels (Fig. 2a). Group II compounds inhibit cell death over a wide range of concentrations (with the IC_{50} in the micromolar range) (Fig. 2b). In contrast, group III compounds exhibit no significant inhibition of cell death (Fig. 2c).

Our data demonstrated that among the group I drugs, four compounds hold special promise as potential neuroprotective drugs because of their lower IC_{50}, and relatively higher maximum protection values. These four, ranked by IC_{50} at nanomolar range from best to worst, are fendiline hydrochloride (7.0 nM), calcimycin (7.0 nM), dipyrone (10.4 nM), and bepridil hydrochloride (128.0 nM). We assume that the other two drugs in group , ritanserin and

azathioprine, display low potential for neurodegeneration because of their very low maximum protection values (26.9 % and 27.6%, respectively), even though their IC_{50} values are lower (49.1 nM and 100.4 nM, respectively) (Fig. 2a).

Among group II, eight additional compounds might exhibit high potential for protection against neurodegeneration (Fig. 2b). These group II compounds, ranked by IC_{50} at micromolar range from best to worst, are parthenolide (1.8 μM), probucol (8.3 μM), methazolamide (9.8 μM), hydroxy-progesterone caproate (11.7 μM), doxycycline hydrochloride (13.4 μM), melatonin (14.8 μM), minocycline hydrochloride (15.5 μM), and N-acetyl-DL-tryptophan (33.1 μM). Mephenytoin, one member of group II, might have a low potential for neuroprotectionn since its maximum protection value is low (23.3%).

None of the six compounds in group III were recommended as drug candidates for neurodegenerative diseases because they exhibit no significant inhibition of cell death and may be toxic (Fig. 2c). We note that the toxic properties of the inhibitor of cytochrome c release gossypol acetic acid complex which is even ranked first in Figure 1, are greater than its beneficial effects, at least when tested on mutant-htt ST14A striatal cell following serum withdrawal at the non-permissive temperature; therefore, it is defined as belonging to group III and can be used as the negative control.

Our study therefore found, for the first time, a class of 12 inhibitors of cytochrome c release among of 21 compounds with therapeutic potential for neurodegeneration. Some of these 12 compounds have already been reported to be protective against cell death or toxicity. Dipyrone (metamizol) has an anti-apoptotic effect on HL-60, Jurkat, and Raji cell lines under a variety of apoptotic induction conditions (Pompeia et al., 2001) and offers long-lasting neuroprotection in a rat ischemia model (Coimbra et al., 1996). The antioxidant probucol reduces HgCl(2) toxicity (Gasso et al., 2001). Doxycycline hydrochloride was previously found to be neuroprotective (Yrjanheikki et al., 1998). Methazolamide and melatonin (N-acetyl-5-methoxytryptamine) have been reported to prevent SH-SY5Y neuroblastoma cell death following oxidative damage (Sarang et al., 2002). Furthermore, minocycline is protective against neurodegeneration; it reduces tissue damage following ischemia and traumatic brain injury delays onset and extends survival in animal models of ALS and HD, and exhibits remarkable neuroprotective properties in experimental models of Parkinson disease and multiple sclerosis (Brundula et al., 2002; Chen et al., 2000; Friedlander, 2003; Wang et al.,

2003; Wu et al., 2002; Yrjanheikki et al., 1998; Zhu et al., 2002). Considering the neuroprotective properties of methazolamide and melatonin in the H_2O_2/SH-SY5Y cellular model of oxidative damage (Sarang et al., 2002) and the similarity (even better) of their IC_{50} values with that of minocycline, we further evaluated the screen by selecting the two compounds in same group II with minocycline for their protective role against neurodegeneration, using minocycline as the positive control.

Methazolamide and melatonin offer neuroprotection in cellular model of ischemia in vitro.

Methazolamide is currently used as a diuretic in humans, having been approved by the FDA as a treatment for glaucoma. At the molecular level, it is a noncompetitive inhibitor of carbonic anhydrase (Lindskog, 1997). However, there are no reports of methazolamide as a potential neuroprotective drug for neurodegenerative diseases. Like methazolamide, melatonin, a pineal-gland hormone implicated in the control of circadian rhythms, efficiently blocks cytochrome c release from purified mitochondria and crosses the blood-brain barrier. Melatonin is currently administered to blind patients, who tolerate high doses without substantial side effects. Because melatonin is known to scavenge free radicals, thereby acting indirectly as an anti-oxidant, it is being investigated as a therapy for ALS, HD, Parkinson disease, and damage from stroke (Reiter et al., 1999; Reiter et al., 2003; Sarang et al., 2002). However, our understanding of the mechanism by which melatonin controls cell death is not complete. We have demonstrated that methazolamide and melatonin are neuroprotective in mutant-htt ST14A striatal cells under a non-permissive temperature shift (Fig. 2b). To further confirm that the inhibition of cell death by the two inhibitors of cytochrome c release is universally effective, we further tested whether they were neuroprotective against a variety of cell death inducers—H_2O_2, NMDA, and OGD—in PCN, an *in vitro* model for both ischemia and cell death, making this model closer to in vivo physiology than are striatal cells transformed by large T antigen. We found that the incubation with different concentrations of methazolamide or melatonin, as with the positive control minocycline (Figure 3a), significantly inhibited H_2O_2 or NMDA, or OGD-induced cell death (*$p < 0.05$, **$p < 0.001$). The data complemented previous findings that melatonin protected primary cultures of rat cortical neurons from cell death (Cazevieille et al., 1997; Yamamoto and Tang, 1998). We thus demonstrated that methazolamide and melatonin offer neuroprotection in an in vitro cellular

model of ischemia caused by a variety of cell death challenges and that they both indeed hold special promise as potential neuroprotective drugs.

The ability of test drugs to block cell death is associated with the ability to protect against loss of $\Delta\Psi m$ in living cells.

It is now apparent that mitochondrial dysfunction with the upstream loss of $\Delta\Psi$m is the key activating event underlying neuronal cell death in neurodegeneration (Friedlander, 2003; Wang et al., 2003). The dissipation of the $\Delta\Psi$m is a physiologic change accompanying programmed cell death (Hengartner, 2000). As an upstream event of PT induction, the dissipation of $\Delta\Psi$m, causing the permeability transition pore in mitochondrial membranes to open, has been implicated as a critical effector of cell death (Tatton and Chalmers-Redman, 1998). Electrostatically charged mitochondria can be visualized in biological tissue by staining. Rh 123 is such a paotentiometric dye already used to monitor mitochondrial function in apoptotic cells (Wang et al., 2003; Zhang et al., 1999). We previously demonstrated that minocycline acts, at least in part, by inhibiting release of cytochrome c and the dissipating the $\Delta\Psi$m (Wang et al., 2003). We aim to evaluate whether the ability of the test drugs to inhibit cell death, similar to that of the positive control minocycline, was consistent with their ability to prevent the loss of $\Delta\Psi$m in living neurons.

Our data indicated that, in association with the increased cell death, the dissipation of $\Delta\Psi$m indeed did increase progressively in PCNs upon OGD challenge (Fig. 3b), and in mutant-htt ST14A striatal cells under a non-permissive temperature shift by a time-dependent mode (Fig. 3c). Methazolamide and melatonin effectively rescued the collapse of $\Delta\Psi$m; Rh 123 staining demonstrated more punctate staining, consistent with normal mitochondrial function (Fig. 3b,c). In contrast, gossypol-acetic acid complex did not prevent the loss of $\Delta\Psi$m upon induction of mutant-htt ST14A striatal cell death (Fig. 3c). The inability of gossypol-acetic acid complex to protect against striatal neuronal death was accompanied by the inability to protect against the loss of membrane potential, and both methazolamide and melatonin blocked the loss of $\Delta\Psi$m, the upstream step of mitochondrial dysfunction. We thus propose that these findings, taken together, suggest that the ability of the test drugs, at least methazolamide, melatonin, minocycline, and gossypol-acetic acid complex, to block cell death

in the cell systems of PCNs and mutant-htt ST14A cells is associated with their ability to prevent loss of ΔΨm.

Methazolamide and melatonin are not inhibitors of the mitochondrial permeability transition (mPT) in isolated mitochondria.

The mitochondrial permeability transition represents a multiprotein complex composed of proteins of the mitochondrial matrix, including the outer and inner membranes (Zoratti et al., 2005). Various pathologic factors such as high concentrations of Ca^{2+}, pro-oxidants, thiol cross-linking reagents, and proapoptotic cytosolic proteins might induce activation of mPT and its irreversible opening. During mitochondrial dysfunction, the loss of ΔΨm is an upstream step; activation of mPT is the next event.

Because of the difficulty of testing mPT in living cells, we evaluated whether methazolamide and melatonin delayed or prevented mPT induced by different agents in isolated liver mitochondria. These challenges included high concentrations of Ca^{2+}, oxidative agents such as organic hydroperoxide tBH, the thiol cross-linking agent PhAsO, thiol oxidant diamide, or their combination (Fig. 3d,e). Neither drug had protective effects on isolated mitochondria under these conditions. The positive control minocycline can block both the loss of ΔΨm in neuronal living cells and activation of mPT on isolated mitochondria (Wang et al., 2003; Zhu et al., 2002), whereas methazolamide and melatonin blocked only the upstream step of mitochondrial dysfunction in neuronal living cells but not the next event of mPT activation in isolated mitochondria; we therefore suggest the mechanisms of action and inhibition of methazolamide are not identical to those of melatonin and minocycline.

Methazolamide, like melatonin, decreases infarct size and neurologic impairment after MCAO.

Minocycline rescues neurons in cellular models of cell death and decreases the volume of the infarct from focal ischemia (Yrjanheikki et al., 1999). Since methazolamide and melatonin offer neuroprotection in cellular model of ischemia in vitro, we further tested whether they have the potential to ameliorate ischemic damage in vivo. After MCAO, the infarct size in mice treated with methazolamide or melatonin was significant reduced as compared with that of DMSO-treated stroke control mice (Fig. 4a,b). Consistent with this reduction, the neurologic impairment scores after MCAO were significantly improved (Fig.

4a,c). Thus, our data demonstrate for the first time that methazolamide offers neuroprotection in models of cerebral stroke. In addition, the protection by melatonin of mice with ischemia-induced stroke complement previous findings that melatonin reduces the volume of cerebral infarction in a rat middle cerebral artery occlusion stroke model (Pei et al., 2003; Reiter et al., 2003).

Caspase-mediated mitochondrial cell death pathways play important roles in neurodegenerative diseases (Beal, 1999; Chan, 2004; Friedlander, 2003; Wang et al., 2003; Zhang et al., 2003b; Zhu et al., 2002), and cytochrome c release and caspase-3 activation have been reported in mice with ischemic damage (Chan, 2004; Kim et al., 2000; Zhang et al., 2003b). Thus, we tested whether methazolamide and melatonin, which inhibited cytochrome c release in isolated mitochondria, prevented cytochrome c release and caspase-3 activation. We found that the significant neuroprotective ability of methazolamide and melatonin was associated with an ability to significantly reduce the release of cytochrome c and activation of caspase-3 in a model of ischemia stroke in vivo (Fig. 4d,e).

Discussion

The 1,040 FDA-approved compounds assembled by NINDS provide a wealth of potentially neuroprotective agents. Because the release of cytochrome c from mitochondria triggers caspase activation, blocking that critical step should derail the cell death program and neurodegenerative diseases. With our screening of inhibition of cytochrome c release in a cell-free system and of cell death in cultured cells, we developed a rapid and reliable assay for selecting potential drugs among many compounds. Our assumption underlying this program of drug discovery is the following: a compound that inhibits the release of cytochrome c induction from isolated mitochondria is likely to prove protective against neurodegeneration. Any of these compounds that prove efficacious in CNS neuronal cell-based systems, with lower IC_{50} values and higher maximum protection, are tested in the animal models of neurodegeneration and eventually tested on human patients with neurodegenerative diseases. Affirming the validity of this premise is the previous finding that two of the potent inhibitors of cytochrome c release in the cell-free system and in cultured cells, minocycline and doxycycline, are protective against neurodegeneration (Brundula et al., 2002; Chen et al., 2000; Friedlander, 2003; Wang et al., 2003; Wu et al., 2002; Yrjanheikki et al., 1998; Zhu et al., 2002). Furthermore, our present study showed that methazolamide and melatonin, two other

cytochrome c release inhibitors, selected on the basis of their activity in the cell-free system and in cultured cells, are protective against cell death and ischemic stroke. Our assay, different with the large scale high through put, provides a rapid small scale drug screen focused on the FDA-approved bioactive compounds. Our study therefore provides a rapid assay for the rational design, screen, and discovery of drugs effective against neurodegeneration.

We screened 21 "hit" inhibitors of cytochrome c release in cultured cells and identified 12 compounds with high potential against neurodegeneration. Among these 12 compounds, methazolamide and melatonin, with excellent safety records in humans and a proven ability to penetrate the blood-brain barrier, were further tested in models of neurodegenerative disease. Methazolamide and melatonin ameliorated neurologic damage in an animal model of cerebral stroke and inhibited neuronal cell death by preventing the loss of loss of ΔΨm. These compounds are therefore attractive candidates for the treatment of acute stroke. Considering that methazolamide and melatonin inhibited polyglutamine-induced cell death in a cellular model of HD, and in PCN, the primary targets of degeneration in HD are the striatal and cortical CNS neurons (Li, 1999; Reiner et al., 1988), these data should assist in the development of therapeutic strategies for chronic neurodegeneration of HD. The FDA-approved drugs methazolamide and melatonin may thus be novel therapy for neurodegeneration. Furthermore, our findings demonstrate that melatonin is significantly effective in blocking striatal and PCN cell death through preventing the loss of ΔΨm and that it has a neuroprotective role in mice with ischemic stroke are complementary to the findings that melatonin protects against neurodegeneration (Feng et al., 2004; Jacob et al., 2002; Pei et al., 2003; Reiter et al., 1999; Tunez et al., 2004; Willis and Armstrong, 1999).

Methazolamide is a noncompetitive inhibitor of carbonic anhydrase. Carbonic anhydrase is a zinc-containing enzyme that catalyzes the reversible hydration of carbon dioxide: $CO_2 + H_2O \longleftrightarrow HCO_3(-) + H^+$. Drugs such as methazolamide and acetazolamide target carbonic anhydrase in the treatment of glaucoma (Lindskog, 1997). However, whether the neuroprotection provided by methazolamide involves carbonic anhydrase is not known. Because this is first report of the potential of methazolamide as a therapy drug for neurodegeneration, and the mechanism of melatonin action is not known in detail, understanding the mechanisms by which these drugs are neuroprotective is of great importance and could contribute to therapies for neurodegenerative diseases. In addition, such knowledge

is useful to choose rational combinations of compounds with different molecular targets. Using this paradigm, in current study can be performed further mechanistic studies of individual test drug. We identify and characterize more compounds as inhibitors of cytochrome c release that are especially promising as potential neuroprotective drugs to slow the progression of neurodegenerative diseases such as cerebral stroke and HD.

Among the 21 inhibitors, there are four antibiotic compounds: calcimycin in group I, minocycline and doxycycline in group II, and metampicillin in group III. Two known neuroprotective agents, minocycline and doxycycline, are both tetracycline-derivatives and have similar chemical structures (Fig. 5). The relationship between the chemical structure of the compounds and their functionality is assessed. Structural analysis showed that three group I compounds, fendiline hydrochloride, ritanserin, bepridil hydrochloride, and one group III compound, meclizine hydrochloride, have a common pharmacophore, with two phenyl rings and one positively charged center (nitrogen) (Fig. 5).

<u>References for Example 1 and Background Section</u>

Beal, M. F. (1999). Biochem Soc Symp *66*, 43-54.

Bouchier-Hayes, L., et al., (2005). J Clin Invest *115*, 2640-2647.

Brundula, V., et al. (2002). Brain *125*, 1297-1308.

Cazevieille, C., et al., (1997). Brain Res *768*, 120-124.

Chan, P. H. (2004). Neurochem Res *29*, 1943-1949.

Chen, M., et al., (2000). Nat Med *6*, 797-801.

Coimbra, C., et al., (1996). Stroke *27*, 1578-1585.

Feng, Z., et al., (2004). J Pineal Res *37*, 129-136.

Friedlander, R. M. (2003). N Engl J Med *348*, 1365-1375.

Gasso, S., et al., (2001). J Neurosci Res *66*, 135-145.

Heemskerk, J., et al., (2002). Meeting of the Neurodegeneration Drug Screening Consortium, 7-8 April 2002, Washington, DC, USA. Trends Neurosci *25*, 494-496.

Hengartner, M. O. (2000). The biochemistry of apoptosis. Nature *407*, 770-776.

Jacob, S., et al. (2002). J Pineal Res *33*, 186-187.

Jones, P. A., et al., (2004). Cell Res *14*, 241-250.

Kandel, E. R., et al., (eds. 2000). Principles of Neural Science, 4th Edition. (McGraw-Hill,

New York).

Kim, G. W., et al., (2000). J Cereb Blood Flow Metab *20*, 1690-1701.

Kumar, U. (2004).Neurosci Res *49*, 29-38.

Li, P., et al., (1997). Cell *91*, 479-489.

Li, X. J. (1999). Mol Neurobiol *20*, 111-124.

Lindskog, S. (1997). Pharmacol Ther *74*, 1-20.

Pei, Z., et al., (2003). Stroke *34*, 770-775.

Pompeia, C., et al., (2001). Int Immunopharmacol *1*, 2173-2182.

Reiner, A., et al., (1988). Proc Natl Acad Sci U S A *85*, 5733-5737.

Reiter, R. J., et al., (1999). Ann N Y Acad Sci *890*, 471-485.

Reiter, R. J., et al., (2003). Ann N Y Acad Sci *993*, 35-47; discussion 48-53.

Rigamonti, D., et al., (2000). J Neurosci *20*, 3705-3713.

Rigamonti, D., et al., (2001). J Biol Chem *276*, 14545-14548.

Sarang, S. S., et al., (2002). Physiol Genomics *11*, 45-52.

Shan Zhu, et al., Nature 417, 74-78 (2 May 2002).

Stavrovskaya, I. G., et al., (2004). J Exp Med *200*, 211-222.

Tatton, W. G., and Chalmers-Redman, R. M. (1998). Ann Neurol *44*, S134-141.

Tunez, I., et al., (2004). J Pineal Res *37*, 252-256.

Wang, X., et al., (2005). J Neurosci *25*, 11645-11654.

Wang, X., et al., (2003). Proc Natl Acad Sci U S A *100*, 10483-10487.

Willis, G. L., and Armstrong, S. M. (1999). Physiol Behav *66*, 785-795.

Wu, D. C., et al., (2002). J Neurosci *22*, 1763-1771.

Yamamoto, H., and Tang, H. (1998). Toxicol Lett *94*, 13-18.

Yrjanheikki, J., et al., (1998). Proc Natl Acad Sci U S A *95*, 15769-15774.

Yrjanheikki, J., et al., (1999). Proc Natl Acad Sci U S A *96*, 13496-13500.

Zhang, D., et al., (1999). J Neurosci Res *58*, 284-292.

Zhang, W., et al., (2003a). Proc Natl Acad Sci U S A In press.

Zhang, W. H., et al., (2003b). Proc Natl Acad Sci U S A *100*, 16012-16017.

Zhu, S., et al., (2002). Nature *417*, 74-78.

Zoratti, M., et al., (2005). Biochim Biophys Acta *1706*, 40-52.

Zou, H., et al., (1997). Cell *90*, 405-413.

Example 2

Methods

Drugs. The drugs were obtained from the 1,040 compounds National Institute of Neurological Disorders and Stroke library by Custom Collection of Microsource Discovery Systems Inc., or were obtained from Sigma.

Mitochondrial Screen. Mouse liver mitochondria were purified following the previously described method[7]. An aliquot of 100 μl of (0.1 mg/ml) mouse liver mitochondrial preparation was preincubated with compounds from the NINDS drug library at a final concentration of 20 μM (diluted with assay medium by 500x from the 10 mM stock solution in DMSO) for 5 min in MRM buffer (250 mM sucrose, 10mM Hepes, pH 7.5, 1mM ATP, 5 mM sodium succinate, 0.08 mM ADP, 2 mM K_2HPO_4, pH7.5) at a concentration of 0.5 mg ml^{-1}. Mitochondria were then incubated with 20 μM $CaCl_2$ at 30°C for 30 min ($CaCl_2$). Mixtures were centrifuged at 10,000 x g at 4°C for 10 min and the supernatant evaluated using a Cytochrome c ELISA kit (R&D systems).

Enzyme-linked immunoabsorbent assay (ELISA). The NINDS library was obtained as 13 kits with 80 compounds in each. A single 96-well ELISA plate was dedicated to each kit of 80 compounds. Drugs were assayed with a separate mitochondrial preparation for each kit. For each sample within a given 96-well plate, the absorbance at 450 nm (a measure of cytochrome c concentration) was corrected by subtracting the background reading at 540 nm. Cytochrome c concentration was calculated by interpolating these values on a standard curve constructed for each plate.

Cell lines and stimulation of cell death. The cell line ST14A were striatal cells conditionally immortalized by transfection with a temperature-sensitive form of the SV40 large T-antigen. ST14A cells stably expressing a mutant huntingtin fragment (N548mu [nt1955–128], mutant-htt ST14A) cells were cultured as described . Mutant-htt ST14A cells were kindly provided by Dr. Elena Cattaneo (University of Milan, Italy). Cells were shifted to the

non-permissive temperature of 37°C in serum-deprived medium (SDM) for the indicated times[6]. All experiments used 2 hours of pre-incubation with test drugs. Culture of primary cerebrocortical neurons (PCNs) (E14-E16) was performed as previously described[5]. Cerebral cortex of mouse embryos at day 15 (E15) were freed from meninges and separated from olfactory bulb and hippocampus. Trypsinized cells were suspended in medium (neurobasal medium (NBM) with 2% (vol/vol) B27 supplement, 2 mM glutamine, 100 units/ml penicillin, and streptomycin (GIBCO)) and seeded at a density of 2 x 10^4 per cm2 on polylysin-coated dishes. Cells were used for experiments on day seven of culture. PCNs were incubated with 1 mM H_2O_2 or 500 μM NMDA for 18–20 hours, or OGD as described previously[5]. Briefly, for OGD treatment, the culture medium was replaced with glucose-free Earle's balanced salt solution with or without different concentrations of test drugs, and the cells were placed in an anaerobic chamber with BBL GasPak Plus (Becton Dickinson), which reduces the oxygen concentration to □100 ppm within 90 min. Control cells were incubated in Earle's balanced salt solution with glucose in a normoxic incubator for the same period. After 3 hours, OGD was terminated by a return to normal culture conditions. Cell death was determined by MTS or LDH assay.

MTS assay. The assay was performed according to the manufacturer's instructions (Promega). Cell viability was assessed on mutant-htt ST14A cells using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTS) assay, a measure of mitochondrial function and thus cell survival.

Lactate dehydrogenase (LDH) assay. The assay was performed according to the manufacturer's instructions (Roche Products). Briefly, reaction mixture (100 μl) was added to conditioned media (100 μl) removed from plates or dishes after centrifugation at 250 x *g* for 10 min. Absorbance of samples at 490 nm was measured in an ELISA reader after 15 min of incubation at room temperature. The same volume of medium was used as the background control.

Western blot. Samples of mouse brain were lysed in RIPA buffer with protease inhibitors[6,7]. Antibody to caspase-3 were purchased from Cell Signaling Technology (Beverly,

MA), antibody to caspase-9 from Cell Signaling Technology (Beverly, MA), antibody to β-actin from Sigma, and secondary antibodies and ECL reagents from Amersham Pharmacia Biotech.

Cellular and tissue fractionation. Mutant-htt ST14A cells and mouse brain cytosolic fractionations were performed as described[5-7]. Briefly, cells or brain samples were homogenized (10mM HEPES, pH 7.4, 250mM sucrose, 10mM KCl, 1.5mM MgCl2, 1mM EDTA, 1mM EGTA, 1mM dithiothreitol (DTT), plus protease inhibitor cocktail. Released cytochrome c or Smac/Diablo was analyzed by Western blot. Antibody to cytochrome c was purchased from PharMingen. Smac/Diablo antibody from Novus Biologicals (Littleton, CO).

Immunocytochemistry and Mitotracker staining. Mutant-htt ST14A cells were treated as indicated on chamber slides. The cells were incubated with 1.5 μM Mitotracker Red CMXRos (Molecular Probes) prepared in prewarmed culture media for 45 min, and fixed in 3.7% paraformaldehyde prepared in prewarmed culture media for 5 min, and then in ice-cold acetone for 5 min. Blocking was done in 5% BSA in PBS for 30 min. Cells then were incubated with antibodies to cytochrome c and incubated with FITC-conjugated secondary antibodies. Deconvoluted images were taken with a Nikon ECLIPSE TE 200 fluorescence microscope and processed by using IP LAB software (Spectra Services, Webster, NY).

Caspase Activity Assays. Cell extracts and enzyme assays were performed with the ApoAlert caspase fluorescent assay kit as described[6,53] and according to the manufacturer's instructions (Clontech). Briefly, 1 x 10^6 mutant htt ST14A cells were harvested in cell lysis buffer, and incubated on ice for 10 min. The cell lysates were centrifuged at 10,000 x g at 4°C for 3 min to precipitate cellular debris, and the supernatants were evaluated for caspase activity by adding caspase substrate and 2x reaction buffer/DTT mixture. Caspase-3-like substrate Ac-DEVD-AFC was from PharMingen, caspase-9-like substrate Ac-LEHD-AFC was from Calbiochem. Released AFC was quantified in a Bio-Rad Versa Fluoro Meter (excitation at 400 nm and emission at 505 nm).

Determination of $\Delta\Psi_m$. Striatal cells and PCNs were treated as indicated with or

without methazolamide, melatonin, dipyrone, or gossypol-acetic acid complex. Rhodamine 123 (Rh 123) staining (Molecular Probes) (2 μM for 5 min) was performed in living cells. Deconvoluted images were taken with a Nikon ECLIPSE TE 200 fluorescence microscope and processed with IP LAB software (Spectra Services, Webster, NY).

Measurement of mitochondrial permeability transition (mPT). Rat liver mitochondria were isolated from 4-month-old male Fisher 344 × Brown Norway F_1 rats by differential centrifugation, as described previously[6,7]. The isolation buffer contained 0.3 M sucrose, 10 mM HEPES, 1 mM EGTA, and 0.5% BSA. The protein content was determined by the Lowry method with bovine serum albumin as a standard. Isolated mitochondria were then incubated in the buffer containing 250 mM sucrose, 10 mM HEPES, pH 7.4, 2.5 mM KH_2PO_4, and 5 mM K^+-succinate or glutamate/malate, mitochondria 1 mg/ml. Mitochondria swelling was induced by Ca^{2+}, tert-butylhydroperoxide (tBH), PhAsO, or diamide and monitored in standard spectroscopic assay at 540 nm. Methazolamide and melatonin were respectively applied to mitochondria. Mitochondrial physiological parameters including membrane potential ($\Delta\Psi$), Ca^{2+} uptake capacity, redox state of pyridine nucleotides and swelling was performed simultaneously on multichannel dye fluorimeter (C&L Instruments, Inc., www.fluorescence.com, Baranov et al., manuscript in preparation). Mitochondrial membrane potential ($\Delta\Psi$) was estimated using TMRM (60 nM) (Molecular probes) fluorescence intensity changes at excitation and emission wavelengths 543 and 590 nm. Mitochondrial Ca^{2+} uptake and release capacity was measured as changes of extramitochondrial [Ca^{2+}] followed by fluorescence intensity of CaGreen-5N (125 nM) (Molecular probes) at excitation and emission wavelengths 482 and 535 nm. Redox state of pyridine nucleotides was followed by measuring NADH autofluorescence at excitation and emission wavelengths 350 and 450 nm. Mitochondrial swelling was measured as a function of light scattering at λ excitation and emission wavelengths 587 nm.

Permanent middle cerebral artery occlusion (MCAO). Focal cerebral ischemia was produced as described previously[5]. Briefly, anesthesia was induced in 6- to 8-week-old C57BL/6J mice (body weight 20-24 gr.) (Jackson Laboratory) with 2% (vol/vol) isoflurane (70% N_2O/30% O_2) and was maintained with a 1-0.5% concentration. Rectal temperature was

maintained between 37.0 and 37.5°C with a heating pad (Harvard Apparatus). Focal cerebral ischemia was induced by an intraluminal 7-0 nylon thread with a silicone tip (180 μm diameter) introduced into the right cervical internal carotid artery. The thread was inserted 9 ± 1.0 mm into the internal carotid artery up to the middle cerebral artery. A laser Doppler perfusion monitor (Perimed AB) was adhered to the right temporal aspect of the animal's skull and used to confirm middle cerebral artery flow disruption. Control animals underwent sham surgery consisting of only anesthesia and carotid artery dissection. All animals were sacrificed after surgery. The animal's brain was removed, and the cerebral hemisphere was retained for either evaluation of infract volume or Western blot analysis. All animal procedures were conducted in accordance with protocols approved by the Harvard Medical School Animal Care Committee.

Drug treatment in MCAO model. Control animals were treated with a normal saline vehicle control containing 0.4% dimethyl-sulfoxide (DMSO-Sigma, St. Louis, MO, USA) and 3% Tween-20 (Sigma, St. Louis, MO, USA). Methazolamide (Sigma, St. Louis, MO, USA) was prepared in normal saline containing 0.4% DMSO and administered by intraperitoneal injection at a dose of 20 mg/kg. Animals were treated either one hour prior (n=8) or 30 min following permanent MCAO (n=8). Melatonin was prepared in normal saline with 3% Tween-20 and administered by intraperitoneal injection either one hour prior (n=8) or 30 min following (n=8) permanent MCAO. Dipyrone (Sigma, St. Louis, MO, USA) was prepared in normal saline and administered by intraperitoneal injection at a dose of 10 mg/kg body weight one hour prior (n=8) permanent MCAO.

Determination of Infarct Volume. After 24 hours of MCAO, the mice were sacrificed. The brains were rapidly removed and chilled for 2 min. Coronal sections (1-mm thick; $n = 7$) were cut with a mouse brain matrix. Each slice was immersed in a saline solution containing 2% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich) at 37°C for 20 min. After staining, each slice was scanned with an HP scanjet 4200C. The stained and unstained areas of the right hemisphere were quantified with imageJ 1.32j (NIH), and these values were used to calculate the volume infarct expressed as a percentage of the lesioned hemisphere.

Neurobehavioral Examination. Mice were assigned a neurobehavioral score at two and 24 hours following MCAO. A previously reported scale was used[20,54]: 0, no neurological deficits; 1, failure to extend the left forepaw; 2, circling to contralateral side; 3, loss of ability to walk or righting reflex; and 4, death before 24 hours.

R6/2 Mice and treatment regimen. R6/2 mice (Jackson Laboratories, Bar Harbor, Maine) were randomly assigned to two groups. At 6 weeks of age, mice were treated with daily intraperitoneal injections of either saline or methazolamide (40 mg/kg or 20 mg/kg, Sigma, St. Louis, Missouri). For all experiments, each group had equal numbers of males and females. Muscle strength and coordination were evaluated weekly using a rotarod (Columbus Instruments). Disease onset was defined as the first day that the mouse failed to remain seven minutes at 15 rpm. Mortality was defined as the age at death or when the mouse was unable to right itself within 30 seconds. Body weight was recorded weekly. At 25 weeks, groups (n=6) of saline-treated and methazolamide-treated R6/2 and littermate wild-type control mice were deeply anesthetized and then transcardially perfused with 2% buffered paraformaldehyde, brains were cryoprotected and serially sectioned (50 μm), stained for Nissl substance using cresyl violet and immunostained using antibodies for mutant Huntingtin and DARP-32. Sections were rinsed in tris-buffered saline with Tween 20 (TBS-T) containing 10% normal goat serum for one hour. Sections were then incubated overnight in TBS-T with 0.1% sodium azide and mouse antihuntingtin (1:500, Chemicon, Temecula, CA) and anti-DARP-32 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA). Sections were rinsed in TBS-T, followed by a 2-3 hour incubation in secondary antibodies. Sections were then rinsed three times in TBS-T. Antibody complexes were visualized using diaminobenzidine. Preabsorbtion with excess target protein, or omission of either primary or secondary antibody, was used to demonstrate antibody specificity and background generated from the detection assay. Tissue sections were examined using a Nikon Eclipse E800 microscope with a Spot RT digital camera.

Stereology/Quantitation. Serial-cut coronal tissue-sections from the most rostral segment of the neostriatum to the level of the anterior commissure (Interaural 5.34 mm/Bregma 1.54 mm to Interaural 3.7 mm/Bregma -0.10 mm), were used for huntingtin aggregate analysis. Unbiased stereological counts of huntingtin-positive aggregates (>1.0 μm) were obtained from

the neostriatum in 6 mice each from methazolamide-treated and saline-treated R6/2 mice at 25 weeks using Neurolucida Stereo Investigator software (Microbrightfield, Colchester, VT). The total areas of the rostral brain, ventricles, and neostriatum were defined in serial sections. Counting frames were randomly sampled within the neostriatum for huntintin aggregate counts. The optical dissector method was employed estimating the number of huntingtin-positive aggregates. Striatal neuron areas were analyzed by microscopic videocapture using a Windows-based image analysis system for area measurement (Optimas, Bioscan Incorporated, Edmonds, WA). The software automatically identifies and measures profiles. All computer identified cell profiles were manually verified as neurons and exported to Microsoft Excel. Experiments were in accordance with protocols approved by the Harvard Medical School Animal Care Committee.

Statistical analysis. Densitometric quantification was performed with the Quantity One Program (Bio-Rad). Statistical significance was evaluated by t-test or ANOVA: p values < 0.05 were considered significant and are indicated (*), and ** indicates p values < 0.001. Neuropathological data were compared by ANOVA or repeated measures of ANOVA and by non-paired Student's t-test (Statview, CA). Drug analysis, including IC_{50} and maximum protection, were performed by GraphPad Prism program.

Results

Screen for inhibitors of cytochrome c release from purified mitochondria.

To search for inhibitors of cytochrome c release, we have developed a cell-free screening assay to identify drugs that inhibit cytochrome c release from mouse-liver mitochondria. Isolated mitochondria are preincubated with the test drug for five minutes. To induce cytochrome c release, the organelles are treated with 20 μM Ca^{++} for 30 minutes at 37°C. After removing the mitochondria by centrifugation, the supernatant is analyzed by an enzyme-linked immunosorbent assay (ELISA) to determine the concentration of cytochrome c. This procedure was used to test all 1,040 compounds in a library assembled by the Neurodegeneration Drug Screening Consortium of the NINDS. Many of these molecules cross the blood-brain barrier, and the majority are FDA-approved drugs already in clinical usage. Experiments were performed in a "double-blind" fashion by observers unaware of each drug's

identity. Fig. 1a shows the distribution of activities among the 80 compounds compromising one of 13 "kits" that constitute the library. Equivalent data were gathered for the 12 other kits. To generate Fig. 1b, the data for each of these 13 sets of compounds are normalized so that the average in each "kit" is the same. The colored curves generated in this manner are then summed to form a grand distribution covering the entire NINDS library (solid black line). Most drugs have little effect on the release of cytochrome c in response to Ca^{2+}- stimulation. However, there are outlying points, indicative of drugs that substantially stimulate or, more significantly, inhibit cytochrome c release.

To choose potentially neuroprotective compounds, we selected those that depress cytochrome c release by more than 1.5 standard deviations below the mean of the grand distribution (*i.e.*, to the left of the dotted line). In the primary screen, 48 compounds were found to suppress cytochrome c release to below this value. In a second screen, 39 of the top candidates were confirmed to be effective when present at 10 and 20 μM (data not shown). We then selected among them on the basis of their current clinical usage and overall ranking in the above screen. In addition, all drugs causing major side effects and those excluded from the CNS by the blood-brain barrier were removed, leaving 21 compounds (Fig. 1c). Thus, our cell-free assay narrowed the list of compounds for investigation in living cells to fewer than 2% of the original list of 1,040. We note that minocycline is the second most potent drug identified using this protocol, and the related antibiotic doxycycline ranks seventh.

Inhibitors of cytochrome c release are protective of cultured cells

Living cells are far more complex than mitochondria, so it must be determined whether compounds that strongly affect the purified organelle are indeed active when administered to cultured cells. Consequently, we tested *in a cellular system* the 21 compounds that most strongly inhibit cytochrome c release from purified mitochondria. In this experiment, each potentially neuroprotective drug was used to treat mutant-htt ST14A cells, a cellular model of HD [6,23,24], and one in which cytochrome c release is known to stimulate the cell death process [6,11]. The stimulus used to induce cell death is shifting the temperature-sensitive cells from 33°C (the permissive temperature) to 37°C (the non-permissive temperature) in serum-deprived medium [6,23,24].

In order to determine the relative potencies of these drugs, we measured the extent of

cell death as a function of drug concentration. Each drug was tested at 7-10 concentrations. The resulting curves (plotted semi-logarithmically) define the IC_{50} and maximum protection afforded by each compound (Fig. 2). The dose-response curves for the 21 drugs can be classified into three broad categories: Compounds in Groups I and II reduce cell death as induced by temperature shift. Those in Group I are active at lower concentrations than the ones in Group II, having values for IC_{50} in the nanomolar range. However, they are toxic at higher concentrations (Fig. 2a). Compounds in Group II inhibit cell death over a wide range of concentrations, but they have values for IC_{50} in the micromolar range (Fig. 2b). By contrast, Group III compounds, though active on purified mitochondria, exhibit no significant inhibition of cell death (Fig. 2c). It is noteworthy that three compounds in Group I – fendiline hydrochloride, ritanserin, and bepridil hydrochloride -- and the Group III compound meclizine hydrochloride all contain two phenyl rings and one positively charged center (nitrogen). This structural motif is reminiscent of a portion of tetraphenylphosphonium (TPP), a compound known to localize to negatively-charged mitochondria.

We found that four of the compounds in Group I hold special promise as potentially neuroprotective drugs. The IC_{50} for these compounds is particularly low, and they offer strong protection at the saturating concentration. They are fendiline hydrochloride, calcimycin, dipyrone, and bepridil hydrochloride, compounds with IC_{50} of 7 nM, 7 nM, 15 nM, and 128 nM, respectively. Ritanserin and azathioprine are unlikely to prove neuroprotective because they fail to provide even 30% protection when present at saturating concentrations. Nevertheless, their low IC_{50} of 50 nM and 124 nM, respectively, relegate them to Group I (Fig. 2a).

Among Group II compounds, ten were found to be potentially protective against neurodegeneration. These compounds are parthenolide (IC_{50} = 1.3 μM), hydroxyprogesterone caproate (2.5 μM), probucol (6.6 μM), methazolamide (9.5 μM), doxycycline hydrochloride (12.4 μM), N-acetyl-DL-tryptophan (12.6 μM), minocyscline hydrochloride (14.4 μM), and melatonin (15.5 μM) (Fig. 2b). Dicyclomine and mephenytoin, another two Group II drugs, never afforded more than 30 % protection to cultured cells, making them poor candidates for a drug with *in vivo* activity.

The remaining five compounds are relegated to Group III. They do not demonstrate significant neuroprotection, perhaps because they possess intrinsic toxicity (Fig. 2c). Gossypol acetic acid is a case in point. Though it is the single most potent inhibitor of cytochrome c

release from purified mitochondria (see Fig. 1), it is toxic to mutant-htt ST14A striatal cells. Consequently, it is classified as a Group III drug. Being useless for *in vivo* therapy, it makes a good negative control in further experiments.

Among the 21 compounds found to inhibit cytochrome c release from isolated mitochondria, 16 (76 %) are protective in a cellular model of cell death. This high rate of success vindicates our assumption that inhibitors of the molecular process are beneficial to cells in culture. Among these compounds, several were already known to protect against cell death. Dipyrone (a.k.a., metamizol) has an anti-apoptotic effect on HL-60, Jurkat, and Raji cell lines under a variety of conditions know to induce apoptosis [25,26]. Ritanserin, a specific antagonist of the 5-HT2 serotonin receptor, reduces ischemic damage during transient global ischemia [27]. The antioxidant probucol reduces HgCl2 toxicity [28]. Doxycycline hydrochloride has documented neuroprotective potency [16]. Methazolamide and melatonin (N-acetyl-5-methoxytryptamine) have been reported to prevent cell death of SH-SY5Y neuroblastoma cells following oxidative damage[29]. Furthermore, minocycline is protective against various acute and chronic afflictions of the CNS. It reduces tissue damage following ischemia and traumatic brain injury, delays onset and extends survival in animal models of ALS and HD, and exhibits remarkable neuroprotective properties in experimental models of Parkinson disease and multiple sclerosis [1,6,7,15-18,30]. Some reports fail to demonstrate protection using minocycline. However, on the balance, minocycline-mediated neuroprotection has been accomplished in a broad verity of models in a number of independent laboratories [31-33]. Of the 16 drugs found to be protective of cultured cells, three were selected for investigation *in vivo*.

Methazolamide and melatonin are neuroprotective of primary cerebrocortical neurons.

We initially focused our attention on drugs in Group II. Among these ten compounds, methazolamide was chosen because it is tolerated by patients and, too a significant extent, crosses the blood-brain barrier. It is currently used as a diuretic in humans, being approved by the FDA for the treatment of glaucoma. At the molecular level, it is a noncompetitive inhibitor of carbonic anhydrase. However, there are no reports of methazolamide being tested for its benefits to patients with neurodegenerative diseases. The pineal-gland hormone melatonin, a drug that is better studied than methazolamide, also holds potential for the treatment of chronic neurological diseases. Though it hasn't been tested in that capacity, melatonin is known to

cross the blood-brain barrier. Because it affects circadian rhythm and can be safely administered at high doses, melatonin is used to treat blind patients. Melatonin scavenges free radicals, making it a *de facto* anti-oxidant. This property of the compound motivated studies of its potential benefits to patients with ALS, HD, and Parkinson disease and to persons who have suffered a stroke. As described above, we found that both methazolamide and melatonin inhibit mitochondrial cytochrome c release and protect mutant htt ST14A cells from cell death upon temperature shift. To confirm the two compounds' ability to inhibit primary neuronal cell death, we evaluated their ability to protect primary cerebrocortical neurons (PCNs) that are challenged with various stresses, *i.e.*, H_2O_2, NMDA, and oxygen glucose deprivation (OGD). Upon treatment with methazolamide and melatonin, we observed statistically significant inhibition of cell death as induced by each of these stimuli (* indicates that $p < 0.05$, ** that $p < 0.001$ in Fig 3). Note that Fig 3 also includes data on minocycline, a compound that serves as a positive control. These data complement a previous finding that melatonin protects primary cultures of rat cortical neurons from cell death [34,35]. Having observed that these drugs increase the survival of cultured cells subjected to cell death stimuli, we proceeded to study what molecular changes they affect.

Inhibition of cytochrome c release and of caspase activation contribute to neuroprotection by methazolamide and melatonin

Our observation that methazolamide is neuroprotective is the first in the literature. It is of interest to determine the compound's effects on the molecular physiology of the cell. The ability of melatonin to inhibit neuronal cell death is well known, but again its effects on subcellular processes are poorly understood. Investigation of mitochondrial cell death pathways, both caspase-dependent and independent, is a promising avenue of research, as they are of documented importance to neurodegeneration [1,5-9,36,37]. In particular, the release of cytochrome c and other apoptogenic factors from these organelles triggers sequential maturation of caspase-9 and caspase-3, as well as caspase-independent cell death events. Disruption of this cascade of molecular events should forestall cell death. Indeed, recent studies have implicated mitochondria in the mechanism by which melatonin exerts its neuroprotective effects [38]. These considerations motivated us to study the changes that methazolamide and melatonin effect on mitochondrial physiology and caspase activation. Bolstering this reasoning

is our earlier observation that the neuroprotective property of minocycline results from its ability to inhibit these very molecular changes [5-7].

The mutant htt ST14A cell line is a useful system in which to pursue the above investigations. Because these conditionally-immortalized striatal cells carry a gene for temperature-sensitive large T antigen of SV40 and another one encoding polyglutamine-expanded huntingtin, they undergo cell death upon transfer to the nonpermissive temperature of 37°C [6,23]. Cytochrome c localization was evaluated by immunocytochemistry using a fluorescence microscope and deconvolution software. Cells were co-labeled with anticytochrome c (Fig. 6a, green in upper panels) and a mitochondrial marker, MitoTracker (Fig. 6a, red in middle panels). Merged images demonstrate localization of cytochrome c immunoreactivity with mitochondria (Fig. 6a, lower panels). Arrows indicate release of cytochrome c from the mitochondria into the cytoplasm as evidenced by a more diffuse staining pattern (Fig. 6a). Methazolamide and melatonin both inhibited cytochrome c release as evidenced by the preservation of punctate staining following temperature shift (Fig. 6a).

Western blotting of cell homogenates reveals that cytoplasmic levels of cytochrome c and Smac/Diablo were elevated by the temperature shift, as is the concentrations of active caspase-3 and -9. Methazolamide and melatonin inhibit both the release of mitochondrial apoptogenic factors and the proteolysis of procaspases (Fig. 6a,b). In parallel studies, the activity of caspase-9 and -3 were measured by their hydrolysis of fluorogenic tetrapeptide substrates. Again, treatment of cells with methazolamide and melatonin decreased their content of mature (and thus enzymatically active) caspase molecules (Fig. 6 c,d). Taken together, these observations indicate that neuroprotection by methazolamide and melatonin results, at least in part, from their inhibition of mitochondrial-dependent pathways of cell death.

Drugs that forestall cell death also slow dissipation of the mitochondrial potential gradient

Dissipation of the electrostatic potential gradient across the mitochondrial membrane ($\Delta\Psi_m$) is part of the mitochondrial dysfunction that triggers the caspase cascade. Consequently, the collapse of $\Delta\Psi_m$ is a critical change underlying the physiological process of neuronal cell death and resulting neurological disease [1,6,12,39]. Presumably, the dissipation of $\Delta\Psi_m$ results from opening of the permeability-transition pore. Certainly, a membrane channel that is large

enough to allow passage of apoptogenic factors would enable the rapid equilibration of protons and other charged particles. To follow the biochemical process, we observed the effects of test drugs on the localization of rhodamine 123 (Rh 123) within the cell. Rh 123 is a fluorescent, hydrophobic, and positively charged molecule, and therefore one that accumulates within electrostatically charged mitochondria. This chemistry causes stained cells to exhibit a punctate pattern of fluorescence except once their mitochondria have become depolarized. Indeed, observation with a fluorescence microscope of cells stained with Rh 123 is commonly used to monitor mitochondrial function in cells undergoing apoptosis [6,40]. As noted above, the neuroprotective properties of minocycline have been linked to the drug's effects on mitochondria, *i.e.*, inhibition of cytochrome c release, compromised mitochondrial swelling, and preservation of $\Delta\Psi_m$ [6,7]. By analogy, should inhibition of mPT be important to the action of methazolamide and melatonin, these drugs should prevent the dissipation of $\Delta\Psi_m$. To validate (or disprove) this hypothesis, we compared drug-treated and control cells using Rh 123 staining and fluorescence microscopy.

Indeed, we found that there is a strong correlation between dissipation of $\Delta\Psi_m$ and increased cell death of PCNs due to OGD (Fig. 7a). The same phenomenon was observed when mutant-htt ST14A striatal cells were shifted to the non-permissive temperature (Fig. 7b). The above technique using the Rh 123 fluorescent stain revealed that both methazolamide and melatonin inhibit the collapse of $\Delta\Psi_m$. Drug treatment preserved the punctate distribution of Rh 123 fluorescence in the presence of a cellular stress, a property that correlates with compound's ability to reduce the extent of cell death. In spite of its ability to inhibit cytochrome c release from purified mitochondria, and corresponding with in the ability to inhibit cell death, gossypolacetic acid complex did not prevent the loss of $\Delta\Psi_m$ upon induction of cell death of mutant-htt ST14A striatal cells (Fig. 7b). Applying the method of comparison and difference, we conclude that the ability of methazolamide and melatonin to maintain $\Delta\Psi_m$ explains, at least in part, how they are able to inhibit cell death.

Methazolamide and melatonin are not inhibitors of the mitochondrial permeability transition (mPT) in isolated mitochondria.

The mitochondrial permeability transition pore consists of a multimeric complex of proteins spanning the inner and outer membranes [41,42]. Its opening is a component of

mitochondrial dysfunction, itself a result of such biochemical stresses as high concentrations of Ca^{2+} ion, oxidizing agents, compounds that cross-link thiols, and proapoptotic cytosolic proteins. The loss of $\Delta\Psi_m$ is an early event in the cell death pathway and an important trigger of the caspase cascade.

There is no satisfactory method for detecting the mitochondrial permeability transition in living cells. Consequently, we investigated the effects of methazolamide and melatonin on this molecular change in purified mitochondria. In these experiments, mPT was induced by Ca^{2+} ions, the oxidating agent organic hydroperoxide tBH, the thiol cross-linking agent PhAsO, the thiol oxidant diamide, or a combination thereof (Fig. 7c,d). Neither drug protected purified mitochondria from these chemical challenges. These observations contrast with the analogous ones using minocycline, a compound that blocks the loss of the mitochondrial membrane potential both *in vitro* and *in vivo* [6,7]. Because their effects on purified mitochondria are dissimilar, these compounds probably benefit cultured cells via different mechanisms.

Neither inhibition of the mPT nor direct effects on mitochondrial physiology appear to underlie the protection imparted by melatonin and methazolamide. Initial studies in isolated liver mitochondrial focused on monitoring swelling as a marker or mPT induction, and tested the agents at a broad dose response range. No effects were seen, suggesting that these agents do not interfere with mPT induction across a broad concentration range. Since work in other systems, such as cultured cerebellar granular neurons, had suggested that interference with mitochondrial physiology through uncoupling and respiratory inhibition could also appear protective, we examined the effects of methazolamide and melatonin on basic physiological properties. Studies were done on a recently adapted fluorescence system (Baranov et al., manuscript in preparation) that allows simultaneous measurement of membrane potential (by following TMRM fluorescence), Ca^{2+} flux (using Calcium Green 5N), NAD^+/NADH redox status (using auto-fluorescence of the NAD^+/NADH couple) and swelling (via light scatter). The system is an improved, fluorescence-based analog of the electrode-based system we have previously used and described[20,43]. Neither methazolamide (Fig. 7E) nor melatonin (Fig. 7F) had any significant effects on $\Delta\Psi_m$, calcium transport, NAD^+/NADH redox status, or swelling. Methazolamide was associated with a slightly increased sensitivity to Ca^{2+}-overload, but this would be expected to, if anything, slightly facilitate cell death, not to be protective. Thus, the data in Figure 7 C-F suggest that melatonin and methazolamide do not mediate their protective

effects by acting upon basic mitochondrial physiology, including mitochondrial redox status and substrate, proton, electron, or Ca^{2+} transport. These data are most consistent with effects mediated outside the inner mitochondrial membrane/mitochondrial matrix, including potential targets such as *bcl-2* family members (e.g., *bax*) and the elements involved in release of pro-apoptotic proteins. Studies are in progress to address these potential mechanisms. The data in Figure 7 C-F also suggest that these agents will likely be free of mitochondrial-based detrimental effects, enhancing their potential utility for clinical applications.

Methazolamide and melatonin decrease cerebral ischemia-induced injury.

Just as it rescues cultured neurons from hypoxia, minocycline decreases the volume of the infarct produced by focal ischemia[44]. We found that methazolamide and melatonin, like minocycline, forestall cell death due to OGD, suggesting that they may be protective in cerebral ischemia. Indeed, pretreatment with methazolamide or melatonin caused mice to develop smaller infarcts following middle cerebral artery occlusion (MCAO) than do animals injected with the vehicle (Fig. 8a,b). In addition, their post-ischemic behavior was less severely impaired as judged by the criteria of Hara *et. al.*[45] (Fig. 8a,c). The two drugs were effective if administered either one hour before or thirty minutes after the onset of ischemia. We note that even though pre-treatment and post-treatment resulted in a similar protection as evaluated by TTC, pre-treatment resulted in a greater degree of protection from a behavioral point (Fig. 8b,c). Considering that TTC is a gross marker of territorial infarction, and does not distinguish functional versus dysfunctional brain that is alive, it is possible that even though the size of the injury is the same based on TTC criteria, that there is a difference in the cerebral territory that is dysfunctional, but not dead depending whether the mice were pre or post treated with melatonin. These observations are the first to demonstrate that methazolamide offers neuroprotection in an animal model of cerebral ischemia. They also confirm that melatonin is beneficial in animal models of cerebral ischemia, this time in mice rather than in rats[46,47].

Like mitochondrial dysfunction, the resulting caspase-mediated cell death pathways are processes important to a broad spectrum of acute and chronic neurodegenerative diseases[1,5-9]. In particular, cytochrome c release and caspase-3 activation are observed in brains of mice that have recently suffered ischemic damage[5,8,48]. Because they are found to be neuroprotective in animal models of stroke, we determined whether methazolamide and melatonin diminish

cytochrome c release and caspase-3 activation as stimulated by such acute insult. Just as was observed in cultured cells challenged with OGD, we found that methazolamide and melatonin reduced cytochrome c release and caspase-3 activation in ischemic tissue (Fig. 8d,e).

Dipyrone rescues cells subjected to OGD and reduces damage from cerebral ischemia

We next evaluated whether dipyrone, a Group I drug as defined in Fig. 2, protects primary cerebral cortical cells that are challenged with OGD. Within its category, dipyrone was found to be the drug that best protects mu htt ST14A cells from cell death upon temperature shift (up to 70 % inhibition of cell death, IC_{50} = 15 nM). Dipyrone is an anti-inflammatory and analgesic drug that readily crosses the blood brain barrier[25,49]. Only a small concentration of the compound was required to efficiently rescue PCNs from cell death due to OGD (up to 81 % maximum inhibition of cell death, IC_{50} = 0.2 nM) (Fig. 9a,b). Similar to methazolamide and melatonin, dipyrone also inhibited the loss of $\Delta\Psi_m$ as induced by OGD (Fig. 7b). In addition, MCAO produced smaller infarcts and caused less severe behavioral changes in mice treated with dipyrone than in those injected with the saline vehicle (Fig. 9c,d).

Methazolamide delays disease onset and mortality in R6/2 mice, an animal model of chronic neurodegeneration

In light of its ability to counter cell death of PCNs and striatal cells, methazolamide is apt to benefit test animals suffering from chronic neurodegenerative diseases. As noted above, we have shown that methazolamide decreases injury following cerebral ischemia (Fig. 8). This property is reminiscent of minocycline's ability to rescue cultured neurons, decrease the impact of ischemic stroke, and slow neurodegeneration in R6/2 mice[6,17,33]. Motivated by these findings in animals that suffered acute injury, we proceeded to investigate methazolamide's ability to slow chronic neurodegeneration. R6/2 mice were chosen as the animal model because they suffer neurodegeneration reminiscent of that in HD. The latter syndrome results from their expression of a human gene encoding the N-terminal portion of polyQ-expanded huntingtin protein[50]. Indeed, R6/2 mice are widely used for studying novel drug therapies for the human disease[17,33,51,52]. Beginning at six weeks of age, R6/2 mice received daily intraperitoneal injections of 20 mg/kg or 40 mg/kg methazolamide. Control R6/2 mice were injected with saline vehicle. Body weight was monitored weekly. Both the test and control

groups were comprised of equal numbers of males and females. In a dose-dependent manner, methazolamide-treatment significantly delayed disease onset in R6/2 mice by 15% and 27% in the 20mg/kg and 40 mg/kg groups respectively when compared to saline-treated controls (Fig. 10a-c). In addition, methazolamide treatment significantly extended their lifespan by 11% (20 mg/kg) and 20% (40 mg/kg) (Fig. 10d). However, the time course of weight loss was not significantly altered (Fig. 10c). Marked neuroprotection at the tissue level was observed in methazolamide-treated R6/2 mice, as evidenced by reduced brain weight loss (Fig. 10e). Consistent with the attenuation of loss of brain weight, gross brain atrophy and bilateral ventricular hypertrophy present in saline treated R6/2 mice was significantly reduced in the methazolamide-treated mice ($F_{(2,18)}$ 15.12 , $*P<0.01$) (Fig. 10f). Parallel to these findings, methazolamide administration also significantly ameliorated the striatal neuronal atrophy present in the saline-treated R6/2 mice (WT littermate control: 136.8 ± 9.8 gm^2; methazolamide-treated R6/2 mice: 105.7 ± 15.7 gm^2; saline-treated R6/2 mice: 57.4 ± 21.6 gm^2; ($F_{(2,18)}$ 21.61, $*p < 0.01$) (Fig. 10f). In addition, since dopamine and cAMP-regulated phosphoprotein (DARP) is present in medium-sized striatal neurons, we performed an immunohistochemical analysis of DARP32-immunopositive neurons in the R6/2 mice. In comparison to the saline-treated R6/2 mice, methazolamide markedly reduced the loss of DARP32 immunoreactive striatal neurons. In addition, while the early and progressive accumulation of huntingtin aggregates is a pathological hallmark in R6/2 mice, methazolamide treatment did not significantly reduce huntingtin-positive striatal aggregates in R6/2 mice (saline-treated R6/2 mice: $5.27 \times 10^6 \pm 1.18$; methazolamide-treated R6/2 mice R6/2 mice: $5.06 \times 10^6 \pm 1.04$; $F_{(2,12)}$ 21.61, $p < 0.22$). The finding of lack of effect of methazolamide treatment on huntingtin aggregates is similar to that observed with other neuroprotective strategies [17,51].

These observations constitute the first report of methazolamide's ability to benefit R6/2 mice. In addition, they provide proof of principle that the screen for inhibitors of cytochrome c release from purified mitochondria is a useful technique for identifying drugs with the potential to slow neurodegeneration. It is our hope that methazolamide will also be of clinical value for the treatment of persons afflicted by neurodegenerative diseases.

Discussion

Among the 1,040 compounds in the NINDS library, there are likely to be

neuroprotective agents. The challenge facing the pharmacological researcher is to identify these active species. Previous screens using this same library have been published demonstrating efficacy in identifying neuroprotective compounds [19-22]. Because the release of cytochrome c from mitochondria triggers caspase activation, blocking that critical step should interfere with the cell death program thereby rescue dying neurons. What is needed is a cell-free assay that measures each compound's ability to affect this molecular process. Purified mitochondria stimulated with Ca^{++} ions provide the requisite *in vitro* system. Released cytochrome c, *i.e.*, that which remains in the supernatant once the organelles have been removed by centrifugation, can easily be detected by an enzyme-linked immunosorbent assay (ELISA). Those compounds that reduce the resulting signal are promising candidates for neuroprotective drugs. Compounds from the resulting "short list" are then assayed for their ability to rescue cultured cells challenged with a cell death stimuli, *e.g.*, OGD or incubation at the nonpermissive temperature. Species that are protective in these cellular systems are further evaluated in trials on animal models. Ultimately, biologically safe and potentially curative agents can be tested on human patients afflicted by both acute neurological injury and chronic neurodegenerative. Minocycline and doxycycline, two compounds known *a priori* to slow neurodegeneration [1,6,7,15-18], are particularly effective at inhibiting the release of cytochrome c from purified mitochondria. These observations are reassuring, as they suggest that the *in vitro* assay has predictive value.

Using this three-tiered selection process -- an assay for activity in a cell-free system, observation of effects on cultured cells, and finally trials in animal models -- we successively narrowed the list of potentially therapeutic compounds. Of the 21 compounds selected in the initial screen, 16 proved protective of cells in culture. Due to their success in the cellular models for acute and chronic injury, three compounds were chosen for trials in test animals. Methazolamide, melatonin, and dipyrone all proved to be beneficial *in vivo*. In light of the fact that all three drugs are in current clinical usage, they should not have prohibitive side effects if used in trials on humans.

Taken together, these experiments have demonstrated that methazolamide, melatonin, and dipyrone are protective in an animal model of acute neurological injury (cerebral infarction). They also demonstrate that at least methazolamide is protective in an animal model of chronic neurodegeneration. The conclusions reached above pertain to three drugs in

particular. The study has larger significance, however. It demonstrates that the *in vitro* assay for cytochrome c release from Ca^{++}-stimulated mitochondria can be used to screen for potentially therapeutic compounds. At the physiological level, it validates targeting that molecular process as a rational approach to designing therapies for acute and chronic neurological diseases.

References for Example 2

1. Friedlander, R.M. Apoptosis and caspases in neurodegenerative diseases. *N Engl J Med* **348**, 1365-75 (2003).

2. Bouchier-Hayes, L., Lartigue, L. & Newmeyer, D.D. Mitochondria: pharmacological manipulation of cell death. *J Clin Invest* **115**, 2640-7 (2005).

3. Newmeyer, D.D. & Ferguson-Miller, S. Mitochondria: releasing power for life and unleashing the machineries of death. *Cell* **112**, 481-90 (2003).

4. Nicholls, D.G. Mitochondrial function and dysfunction in the cell: its relevance to aging and aging-related disease. *Int J Biochem Cell Biol* **34**, 1372-81 (2002).

5. Zhang, W.H. et al. Fundamental role of the Rip2/caspase-1 pathway in hypoxia and ischemia-induced neuronal cell death. *Proc Natl Acad Sci U S A* **100**, 16012-7 (2003).

6. Wang, X. et al. Minocycline inhibits caspase-independent and -dependent mitochondrial cell death pathways in models of Huntington's disease. *Proc Natl Acad Sci U S A* **100**, 10483-7 (2003).

7. Zhu, S. et al. Minocycline inhibits cytochrome c release and delays progression of amyotrophic lateral sclerosis in mice. *Nature* **417**, 74-8. (2002).

8. Chan, P.H. Mitochondria and neuronal death/survival signaling pathways in cerebral ischemia. *Neurochem Res* **29**, 1943-9 (2004).

9. Beal, M.F. Mitochondria, NO and neurodegeneration. *Biochem Soc Symp* **66**, 43-54 (1999).

10. Bernardi, P., Scorrano, L., Colonna, R., Petronilli, V. & Di Lisa, F. Mitochondria and cell death. Mechanistic aspects and methodological issues. *Eur J Biochem* **264**, 687-701 (1999).

11. Rigamonti, D. et al. Huntingtin's neuroprotective activity occurs via inhibition of procaspase-9 processing. *J Biol Chem* **276**, 14545-8 (2001).

12. Hengartner, M.O. The biochemistry of apoptosis. *Nature* **407**, 770-6 (2000).

13. Li, P. et al. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91**, 479-89. (1997).

14. Zou, H., Henzel, W.J., Liu, X., Lutschg, A. & Wang, X. Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* **90**, 405-13. (1997).

15. Brundula, V., Rewcastle, N.B., Metz, L.M., Bernard, C.C. & Yong, V.W. Targeting leukocyte MMPs and transmigration: minocycline as a potential therapy for multiple sclerosis. *Brain* **125**, 1297-308 (2002).

16. Yrjanheikki, J., Keinanen, R., Pellikka, M., Hokfelt, T. & Koistinaho, J. Tetracyclines inhibit microglial activation and are neuroprotective in global brain ischemia. *Proc Natl Acad Sci U S A* **95**, 15769-74. (1998).

17. Chen, M. et al. Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease. *Nat Med* **6**, 797-801. (2000).

18. Wu, D.C. et al. Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson disease. *J Neurosci* **22**, 1763-71. (2002).

19. Aiken, C.T., Tobin, A.J. & Schweitzer, E.S. A cell-based screen for drugs to treat Huntington's disease. *Neurobiol Dis* **16**, 546-55 (2004).

20. Stavrovskaya, I.G. et al. Clinically approved heterocyclics act on a mitochondrial target and reduce stroke-induced pathology. *J Exp Med* **200**, 211-22 (2004).

21. Rothstein, J.D. et al. Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. *Nature* **433**, 73-7 (2005).

22. Wang, W. et al. Compounds blocking mutant huntingtin toxicity identified using a Huntington's disease neuronal cell model. *Neurobiol Dis* **20**, 500-8 (2005).

23. Rigamonti, D. et al. Wild-type huntingtin protects from apoptosis upstream of caspase-3. *J Neurosci* **20**, 3705-13. (2000).

24. Wang, X. et al. Dysregulation of receptor interacting protein-2 and caspase recruitment domain only protein mediates aberrant caspase-1 activation in Huntington's disease. *J Neurosci* **25**, 11645-54 (2005).

25. Pompeia, C., Boaventura, M.F. & Curi, R. Antiapoptotic effect of dipyrone on HL-60, Jurkat and Raji cell lines submitted to UV irradiation, arachidonic acid and cycloheximide treatments. *Int Immunopharmacol* **1**, 2173-82 (2001).

26. Coimbra, C., Drake, M., Boris-Moller, F. & Wieloch, T. Long-lasting neuroprotective

effect of postischemic hypothermia and treatment with an anti-inflammatory/antipyretic drug. Evidence for chronic encephalopathic processes following ischemia. *Stroke* **27**, 1578-85 (1996).

27. Globus, M.Y., Wester, P., Busto, R. & Dietrich, W.D. Ischemia-induced extracellular release of serotonin plays a role in CA1 neuronal cell death in rats. *Stroke* **23**, 1595-601 (1992).

28. Gasso, S. et al. Antioxidant compounds and Ca(2+) pathway blockers differentially protect against methylmercury and mercuric chloride neurotoxicity. *J Neurosci Res* **66**, 135-45 (2001).

29. Sarang, S.S. et al. Discovery of molecular mechanisms of neuroprotection using cellbased bioassays and oligonucleotide arrays. *Physiol Genomics* **11**, 45-52 (2002).

30. Sanchez Mejia, R.O., Ona, V.O., Li, M. & Friedlander, R.M. Minocycline reduces traumatic brain injury-mediated caspase-1 activation, tissue damage, and neurological dysfunction. *Neurosurgery* **48**, 1393-9; discussion 1399-401 (2001).

31. Smith, D.L. et al. Minocycline and doxycycline are not beneficial in a model of Huntington's disease. *Ann Neurol* **54**, 186-96 (2003).

32. Hersch, S., Fink, K., Vonsattel, J.P. & Friedlander, R.M. Minocycline is protective in a mouse model of Huntington's disease. *Ann Neurol* **54**, 841; author reply 842-3 (2003).

33. Stack, E.C. et al. Combination therapy using minocycline and coenzyme Q10 in R6/2 transgenic Huntington's disease mice. *Biochim Biophys Acta* **1762**, 373-80 (2006).

34. Cazevieille, C., Safa, R. & Osborne, N.N. Melatonin protects primary cultures of rat cortical neurones from NMDA excitotoxicity and hypoxia/reoxygenation. *Brain Res* **768**, 120-4 (1997).

35. Yamamoto, H. & Tang, H. Effects of 2-amino-7-phosphonohepatanoic acid, melatonin or NG-nitro-L-arginine on cyanide or N-methyl-D-aspartate-induced neurotoxicity in rat cortical cells. *Toxicol Lett* **94**, 13-8 (1998).

36. Li, L.Y., Luo, X. & Wang, X. Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature* **412**, 95-9. (2001).

37. Yu, S.W. et al. Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. *Science* **297**, 259-63. (2002).

38. Leon, J., Acuna-Castroviejo, D., Escames, G., Tan, D.X. & Reiter, R.J. Melatonin mitigates mitochondrial malfunction. *J Pineal Res* **38**, 1-9 (2005).

39. Tatton, W.G. & Chalmers-Redman, R.M. Mitochondria in neurodegenerative apoptosis: an opportunity for therapy? *Ann Neurol* **44**, S134-41 (1998).

40. Zhang, D., Berry, M.D., Paterson, I.A. & Boulton, A.A. Loss of mitochondrial membrane potential is dependent on the apoptotic program activated: prevention by R-2HMP. *J Neurosci Res* **58**, 284-92 (1999).

41. Zoratti, M., Szabo, I. & De Marchi, U. Mitochondrial permeability transitions: how many doors to the house? *Biochim Biophys Acta* **1706**, 40-52 (2005).

42. Crompton, M., Virji, S., Doyle, V., Johnson, N. & Ward, J.M. The mitochondrial permeability transition pore. *Biochem Soc Symp* **66**, 167-79 (1999).

43. Krasnikov, B.F. et al. Comparative kinetic analysis reveals that inducer-specific ion release precedes the mitochondrial permeability transition. *Biochim Biophys Acta* **1708**, 375-92 (2005).

44. Yrjanheikki, J. et al. A tetracycline derivative, minocycline, reduces inflammation and protects against focal cerebral ischemia with a wide therapeutic window. *Proc Natl Acad Sci U S A* **96**, 13496-500 (1999).

45. Hara, H. et al. Inhibition of interleukin 1beta converting enzyme family proteases reduces ischemic and excitotoxic neuronal damage. *Proc Natl Acad Sci U S A* **94**, 2007-12 (1997).

46. Pei, Z., Pang, S.F. & Cheung, R.T. Administration of melatonin after onset of ischemia reduces the volume of cerebral infarction in a rat middle cerebral artery occlusion stroke model. *Stroke* **34**, 770-5 (2003).

47. Reiter, R.J. et al. Melatonin ameliorates neurologic damage and neurophysiologic deficits in experimental models of stroke. *Ann N Y Acad Sci* **993**, 35-47; discussion 48-53 (2003).

48. Kim, G.W., Sugawara, T. & Chan, P.H. Involvement of oxidative stress and caspase-3 in cortical infarction after photothrombotic ischemia in mice. *J Cereb Blood Flow Metab* **20**, 1690-701 (2000).

49. Cohen, O., Zylber-Katz, E., Caraco, Y., Granit, L. & Levy, M. Cerebrospinal fluid and plasma concentrations of dipyrone metabolites after a single oral dose of dipyrone. *Eur J Clin Pharmacol* **54**, 549-53 (1998).

50. Mangiarini, L. et al. Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. *Cell* **87**, 493-506. (1996).

51. Ona, V.O. et al. Inhibition of caspase-1 slows disease progression in a mouse model of

Huntington's disease. *Nature* **399**, 263-7. (1999).

52. Li, J.Y., Popovic, N. & Brundin, P. The use of the R6 transgenic mouse models of Huntington's disease in attempts to develop novel therapeutic strategies. *NeuroRx* **2**, 447-64 (2005).

53. Wang, X. et al. Protective role of Cop in Rip2/caspase-1/caspase-4-mediated HeLa cell death. *Biochim Biophys Acta* **1762**, 742-54 (2006).

54. Friedlander, R.M. et al. Expression of a dominant negative mutant of interleukin-1 beta converting enzyme in transgenic mice prevents neuronal cell death induced by trophic factor withdrawal and ischemic brain injury. *J Exp Med* **185**, 933-40. (1997).

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

All references, including patent documents, disclosed herein are incorporated by reference in their entirety.

What is claimed:

1. A method for preventing or treating a cell-death-associated disease or disorder in a subject, comprising:

administering to a subject in need of such treatment an effective amount of a Group A and/or a Group B cytochrome c release-inhibiting compound to treat the cell death-associated disease or disorder, wherein the subject is otherwise free of indications for treatment with the Group A or Group B cytochrome c release-inhibiting compound that is administered.

2. The method of claim 1, wherein a group A cytochrome c release-inhibiting compound is administered.

3. The method of claim 2, wherein the Group A cytochrome c release-inhibiting compound is calcimycin, or an analog, derivative, or variant thereof that inhibits cytochrome c release.

4. The method of claim 2, wherein the Group A cytochrome c release-inhibiting compound is dipyrone, or an analog, derivative, or variant thereof that inhibits cytochrome c release.

5. The method of claim 2, wherein the Group A cytochrome c release-inhibiting compound is parthenolide, or an analog, derivative, or variant thereof that inhibits cytochrome c release.

6. The method of claim 2, wherein the Group A cytochrome c release-inhibiting compound is hydroxyprogesterone caproate, or an analog, derivative, or variant thereof that inhibits cytochrome c release.

7. The method of claim 2, wherein the Group A cytochrome c release-inhibiting compound is mephenytoin, or an analog, derivative, or variant thereof that inhibits cytochrome c release.

8. The method of claim 2, wherein the Group A cytochrome c release-inhibiting compound is N-acetyl-DL-tryptophan, or an analog, derivative, or variant thereof that inhibits cytochrome c release.

9. The method of claim 1, wherein the cell death-associated disease or disorder is Alzheimer's disease, Parkinson's disease; Huntington's disease; ALS, epilepsy; stroke, head trauma; spinal cord injury; perinatal hypoxic injury; retinal degeneration; cerebral or other end-organ ischemia inducing cell death associated with a neurosurgical, vascular, cardiac, or transplantation surgery; myocardial infarction, or diabetes.

10. The method of claim 1, wherein the subject is human.

11. The method of claim 1, wherein the cytochrome c release-inhibiting compound is linked to a targeting molecule.

12. The method of claim 11 wherein the targeting molecule's target is a neuronal cell.

13. The method of claim 1, wherein the cytochrome c release-inhibiting compound is administered prophylactically to a subject at risk of having a cell death-associated disease or disorder.

14. The method of claim 1, wherein the cytochrome c release-inhibiting compound is administered in combination with an additional drug for treating a cell death-associated disease or disorder.

15. The method of claim 2, further comprising administering a Group B cytochrome c release-inhibiting compound.

16. The method of claim 15 wherein the Group B cytochrome c release-inhibiting compound is fendiline hydrochloride, ritanserin, azathioprine, bepridil hydrochloride, probucol, methoazolamide, doxycycline hydrochloride, melatonin, or minocycline hydrochloride, or an analog, derivative, or variant of fendiline hydrochloride, ritanserin, azathioprine, bepridil hydrochloride, probucol, methoazolamide, doxycycline hydrochloride, melatonin, or minocycline hydrochloride that inhibits cytochrome c release.

17. A method of evaluating the effect of candidate pharmacological agents on cytochrome c release, comprising:

contacting a cell-free sample comprising mitochondria with a candidate pharmacological agent;

determining the effect of the candidate pharmacological agent on the level of cytochrome c release in the cell-free sample relative to the level of cytochrome c release in a control cell-free sample not contacted with the candidate pharmacological agent, wherein a relative decrease in the level of cytochrome c release in the cell-free sample as compared with the level in the control cell-free sample indicates the inhibition of cytochrome c release and/or cell death by the candidate pharmacological agent.

18. The method of claim 17, wherein the mitochondria are contacted with calcium in an amount effective to include cytochrome c release in a control sample.

19. The method of claim 17, wherein the amount of cytochrome c release is determined using an ELISA assay.

20. The method of claim 17, wherein a relative decrease in the level of cytochrome c release in the cell-free sample contacted with the candidate pharmacological agent compared to the level of cytochrome c release in the control cell-free sample indicates the candidate pharmacological agent is a cytochrome c release-inhibiting compound.

21. A kit for treating a subject in accordance with the method of claim 1, comprising

a package housing a first container containing at least one dose of a Group A or a Group B cytochrome c release-inhibiting compound, and

instructions for using the cytochrome c release-inhibiting compound in the prevention and/or treatment of an cell death associated disease or disorder.

22. The kit of claim 21, wherein the container contains at least one dose of a Group A cytochrome c release-inhibiting compound.

23. The kit of claim 22, wherein the Group A cytochrome c release-inhibiting compound is calcimycin, or an analog, derivative, or variant thereof that inhibits cytochrome c release.

24. The kit of claim 22, wherein the Group A cytochrome c release-inhibiting compound is dipyrone, or an analog, derivative, or variant thereof that inhibits cytochrome c release.

25. The kit of claim 22, wherein the Group A cytochrome c release-inhibiting compound is parthenolide, or an analog, derivative, or variant thereof that inhibits cytochrome c release.

26. The kit of claim 22, wherein the Group A cytochrome c release-inhibiting compound is hydroxyprogesterone caproate, or an analog, derivative, or variant thereof that inhibits cytochrome c release.

27. The kit of claim 22, wherein the Group A cytochrome c release-inhibiting compound is mephenytoin, or an analog, derivative, or variant thereof that inhibits cytochrome c release.

28. The kit of claim 22, wherein the Group A cytochrome c release-inhibiting compound is N-acetyl-DL-tryptophan, or an analog, derivative, or variant thereof that inhibits cytochrome c release.

29. The kit of claim 21, wherein the cell death-associated disease or disorder is Alzheimer's disease, Parkinson's disease; Huntington's disease; ALS, epilepsy; stroke, head trauma; spinal cord injury; perinatal hypoxic injury; retinal degeneration; cerebral or other end-organ ischemia

inducing cell death associated with a neurosurgical, vascular, cardiac, or transplantation surgery; myocardial infarction; or diabetes.

30. The kit of claim 22, further comprising a container containing at least one dose of a Group B cytochrome c release-inhibiting compound, wherein the compound is fendiline hydrochloride, ritanserin, azathioprine, bepridil hydrochloride, probucol, methoazolamide, doxycycline hydrochloride, melatonin, or minocycline hydrochloride, or an analog, derivative, or variant of fendiline hydrochloride, ritanserin, azathioprine, bepridil hydrochloride, probucol, methoazolamide, doxycycline hydrochloride, melatonin, or minocycline hydrochloride that inhibits cytochrome c release and instructions for using the compound for prevention and/or treatment of a cell death associated disease or disorder.

31. The kit of claim 21, wherein the cytochrome c release-inhibiting compound is formulated for delivery to neuronal cells.

32. The kit of claim 21, wherein the cytochrome c release-inhibiting compound is formulated for sustained release.

33. The kit of claim 21, wherein the cytochrome c release-inhibiting compound is lyophilized.

34. A composition comprising
a Group A cytochrome c release-inhibiting compound and
a Group B cytochrome c release-inhibiting compound.

35. The composition of claim 34, wherein the Group A cytochrome c release-inhibiting compound is calcimycin, dipyrone, parthenolide, hydroxyprogesterone caproate, mephenytoin, or N-acetyl-DL-tryptophan, or an analog, derivative, or variant of calcimycin, dipyrone, parthenolide, hydroxyprogesterone caproate, mephenytoin, or N-acetyl-DL-tryptophan that inhibits cytochrome c release.

36. The composition of claim 34, wherein the Group B cytochrome c release-inhibiting compound is fendiline hydrochloride, ritanserin, azathioprine, bepridil hydrochloride, probucol, methoazolamide, doxycycline hydrochloride, melatonin, or minocycline hydrochloride, or an analog, derivative, or variant of fendiline hydrochloride, ritanserin, azathioprine, bepridil hydrochloride, probucol, methoazolamide, doxycycline hydrochloride, melatonin, or minocycline hydrochloride that inhibits cytochrome c release.

37. The composition of claim 34, wherein the cytochrome c release-inhibiting compound is linked to a targeting molecule.

38. The composition of claim 37, wherein the targeting molecule's target is a neuronal cell.

39. The composition of claim 34, wherein the cytochrome c release-inhibiting compound is formulated for implantation, mucosal administration, injection, inhalation, or oral administration.

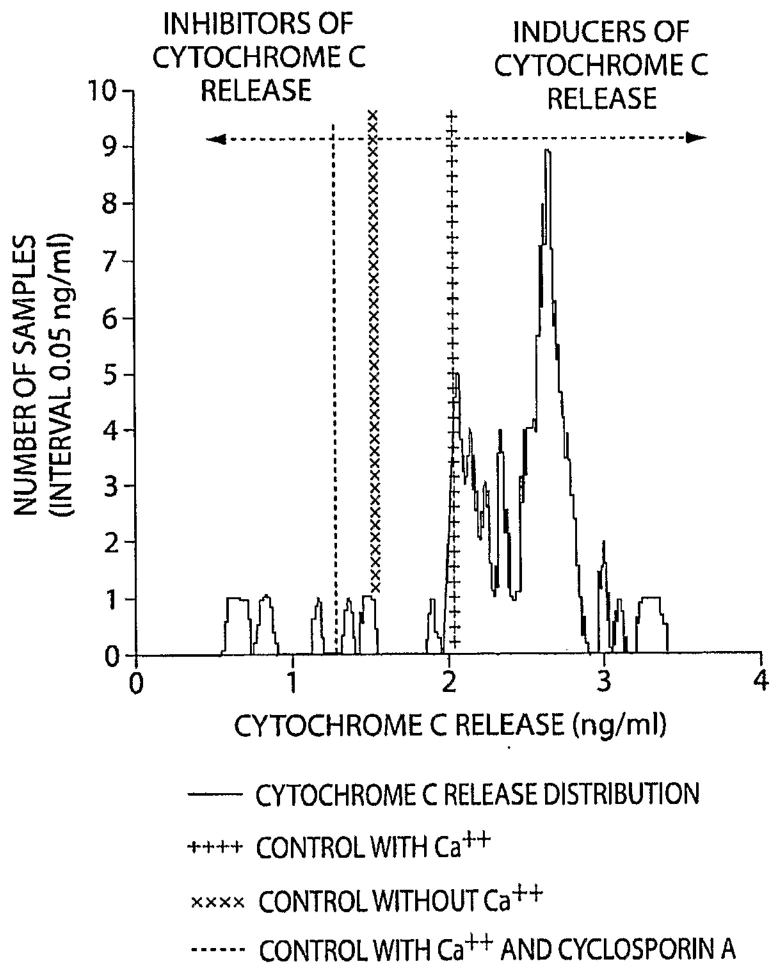


Fig. 1A

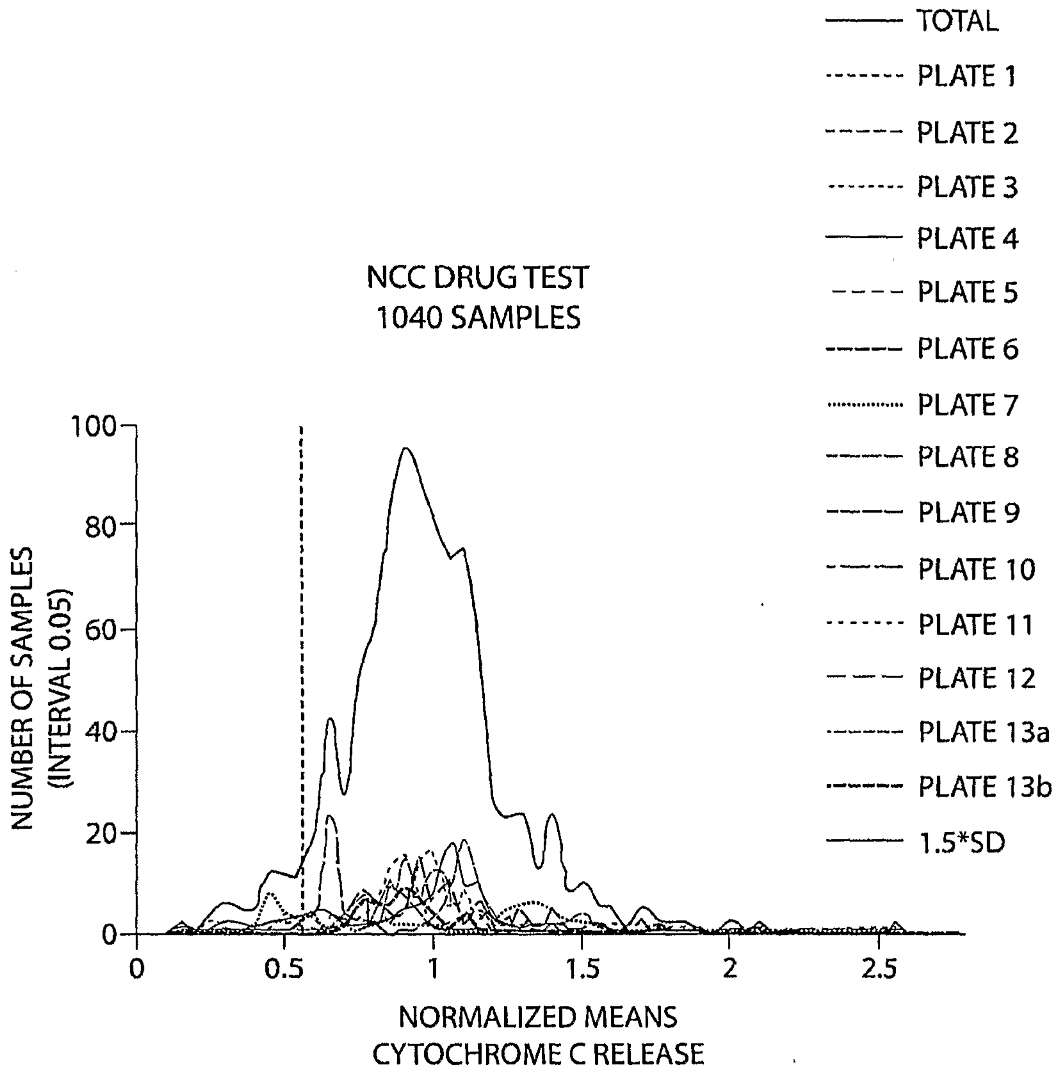
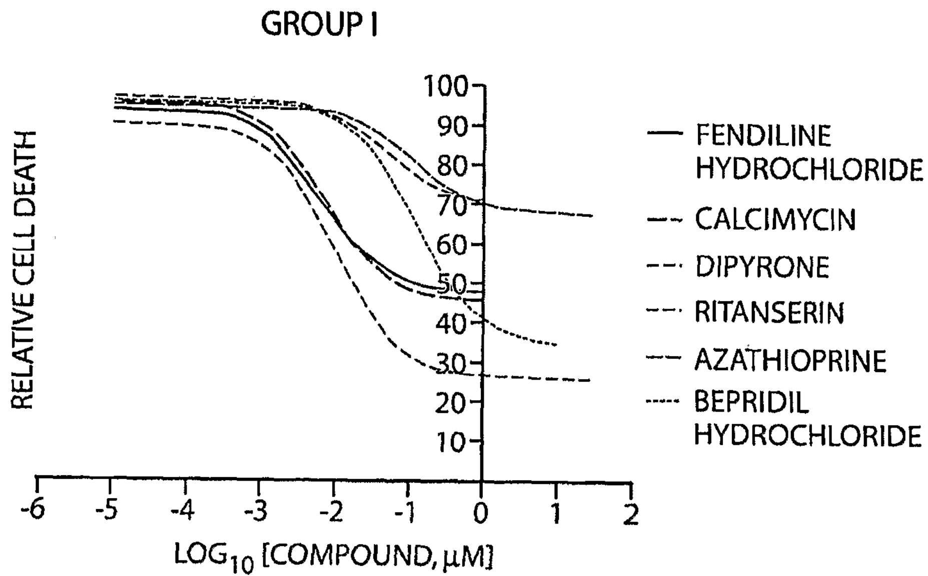


Fig. 1B

RANK	COMPOUND	CLINICAL USAGE
1.	GOSSYPOL ACETIC ACID COMPLEX	ANTI-INFLAMMATORY, ANTI-CANCER AGENT; ANTI-VIRAL AGENT, CONTRACEPTIVE
2.	MINOCYCLINE HYDROCHLORIDE	ANTIBIOTIC (TE TRAC YCLINE-DERIVATIVE WHICH CROSSES BLOOD-BRAIN BARRIER)
3.	HYDROXYPROGESTERONE CAPROATE	HORMONE THERAPY
4.	BEPRIDIL HYDROCHLORIDE	ANTI-ANGINAL AGENT
5.	MECLIZINE HYDROCHLORIDE	ANTAGONIST TO HISTAMINE H1 RECEPTOR
6.	FENDILINE HYDROCHLORIDE	NON-SELECTIVE BLOCKER OF CALCIUM CHANNELS
7.	DOXYCYCLINE HYDROCHLORIDE	ANTIBIOTIC (TETRACYCLINE-DERIVATIVE WHICH CROSSES BLOOD-BRAIN BARRIER)
8.	RITANSERIN	ANTAGONIST TO SERATONIN S2 RECEPTOR
9.	PROBUCOL	DEPRESSANT OF CHOLESTEROL LEVELS
10.	MEPHENYTOIN	ANTI-CONVULSANT
11.	CALCIMYCIN	ANTIBIOTIC; TREATMENT FOR HYPERPARATHYOIDISM
12.	METAMPICILLIN (SODIUM SALT)	ANTIBIOTIC (AMPICILLIN-DERIVATIVE WHICH CROSSES BLOOD-BRAIN BARRIER)
13.	ACETYLTRYPTOPHAN	ANTIDEPRESSANT
14.	MELATONIN	PINEAL GLAND HORMONE USED TO ADJUST BIOLOGICAL CLOCK
15.	LOBELINE SULFATE	RESPIRATORY STIMULANT USED DURING SMOKING CESSATION
16.	BESERAZIDE HYDROCHLORIDE	THERAPY FOR PARKINSON'S DISEASE
17.	AZATHIOPRINE	IMMUNO-SUPPRESSANT; TREATMENT FOR RHEUMATISM
18.	PARTHENOLIDE	ANTI-INFLAMMATORY AGENT
19.	METHAZOLAMIDE	INHIBITOR OF CARBONIC ANHYDRASE USED IN THE TREATMENT OF GLAUCOMA
20.	DIPYRONE	NONSTEROIDAL ANTI-INFLAMMATORY ANTI-PYRETIC AGENT
21.	DICYCLOMINE HYDROCHLORIDE	ANTAGONIST OF ACETYLCHOLINE RECEPTORS

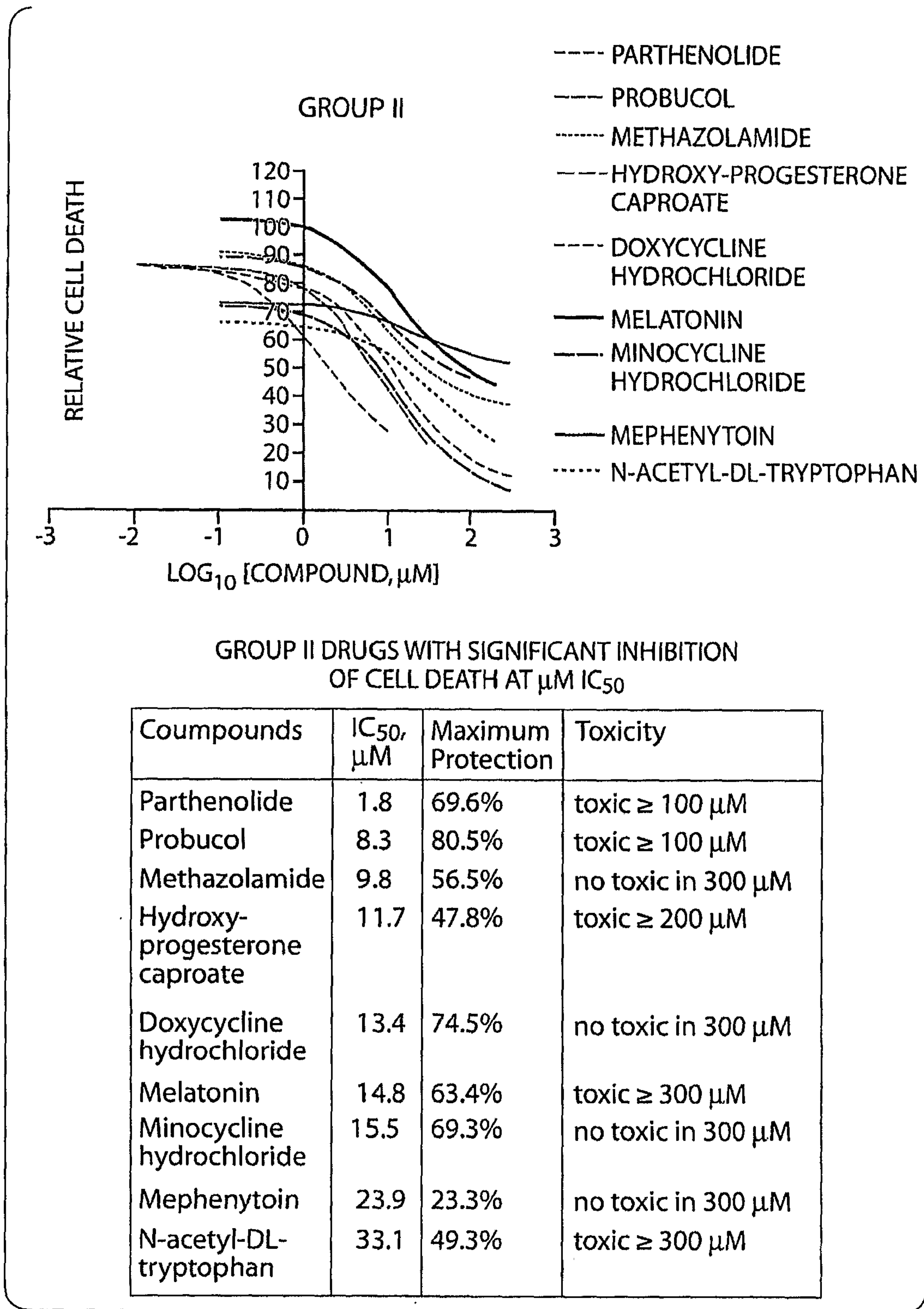
Fig. 1C



GROUP I DRUGS WITH SIGNIFICANT INHIBITION OF CELL DEATH AT nM IC_{50}

Coumpounds	IC_{50}, nM	Maximum Protection	Toxicity
Feudiline hydrochloride	6.7	46.9%	toxic ≥ 100 µM
Calcimycin	7.0	51.6%	toxic ≥ 10 µM
Dipyrone	10.4	64.7%	toxic ≥ 30 µM
Ritanserin	49.1	26.9%	toxic ≥ 30 µM
Azathioprine	100.4	27.6%	toxic ≥ 100 µM
Bepridil hydrochloride	128.0	61.7%	toxic ≥ 100 µM

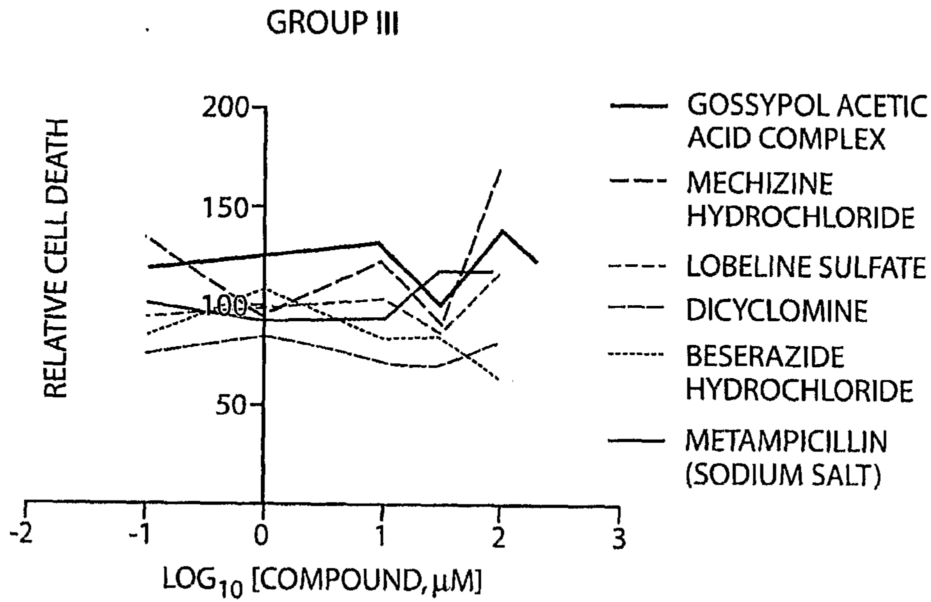
Fig. 2A



GROUP II DRUGS WITH SIGNIFICANT INHIBITION OF CELL DEATH AT µM IC_{50}

Coumpounds	IC_{50}, µM	Maximum Protection	Toxicity
Parthenolide	1.8	69.6%	toxic ≥ 100 µM
Probucol	8.3	80.5%	toxic ≥ 100 µM
Methazolamide	9.8	56.5%	no toxic in 300 µM
Hydroxy-progesterone caproate	11.7	47.8%	toxic ≥ 200 µM
Doxycycline hydrochloride	13.4	74.5%	no toxic in 300 µM
Melatonin	14.8	63.4%	toxic ≥ 300 µM
Minocycline hydrochloride	15.5	69.3%	no toxic in 300 µM
Mephenytoin	23.9	23.3%	no toxic in 300 µM
N-acetyl-DL-tryptophan	33.1	49.3%	toxic ≥ 300 µM

Fig. 2B



GROUP III DRUGS WITH SIGNIFICANT INHIBITION OF CELL DEATH

Coumpounds	Toxicity
Gossypol acetic acid complex	toxic ≥ 0.1 µM
Mechizinc hydrochloride	toxic ≥ 100 µM
Metampicillin (Sodium Salt)	toxic ≥ 200 µM
Lobeline sulfate	toxic ≥ 100 µM
Beserazide hydrochloride	no toxic in100 µM
Dicyclomine	no toxic in100 µM

Fig. 2C

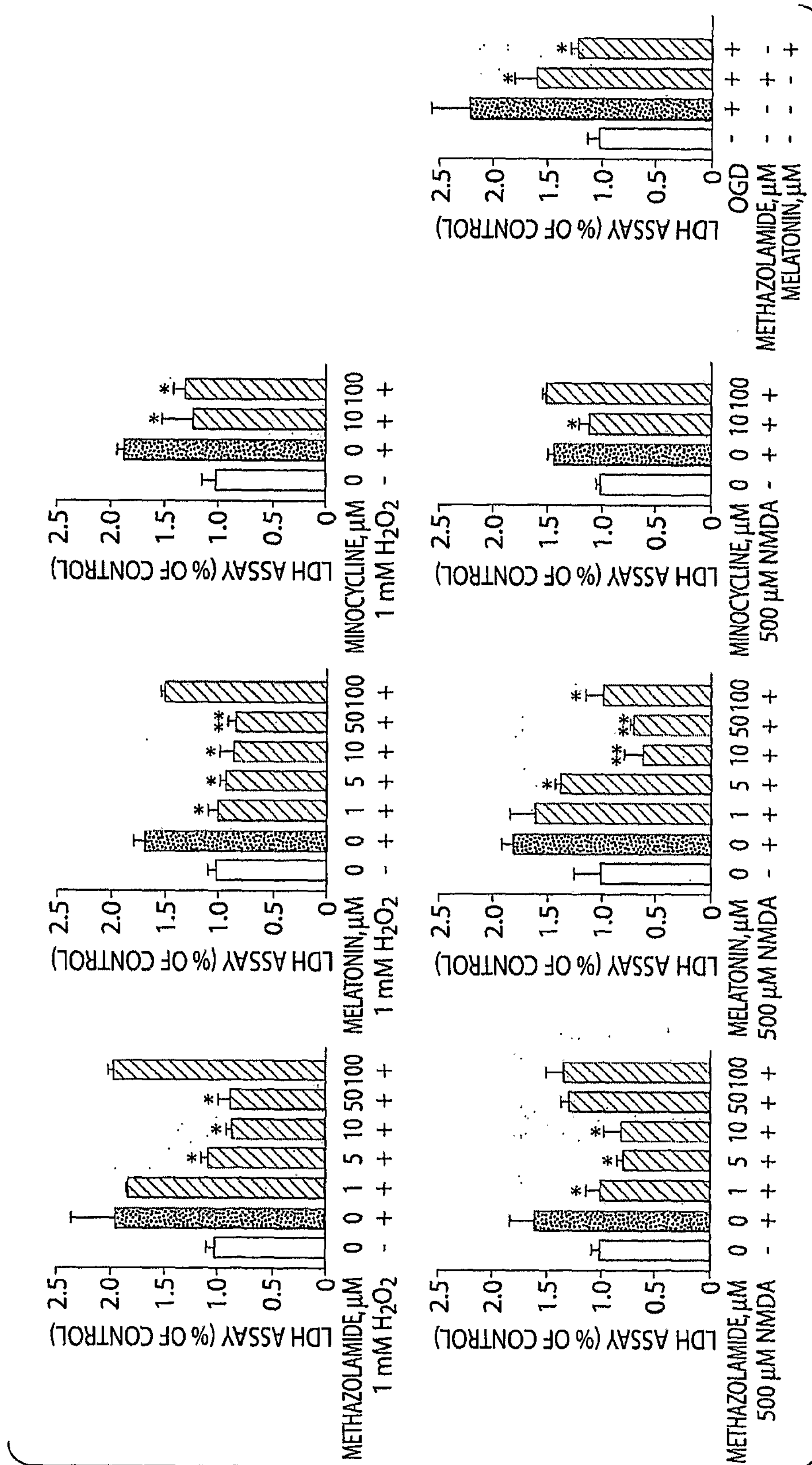


Fig. 3A

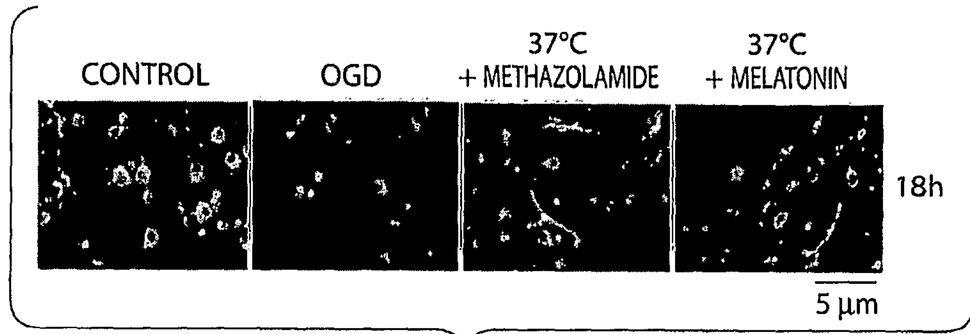


Fig. 3B

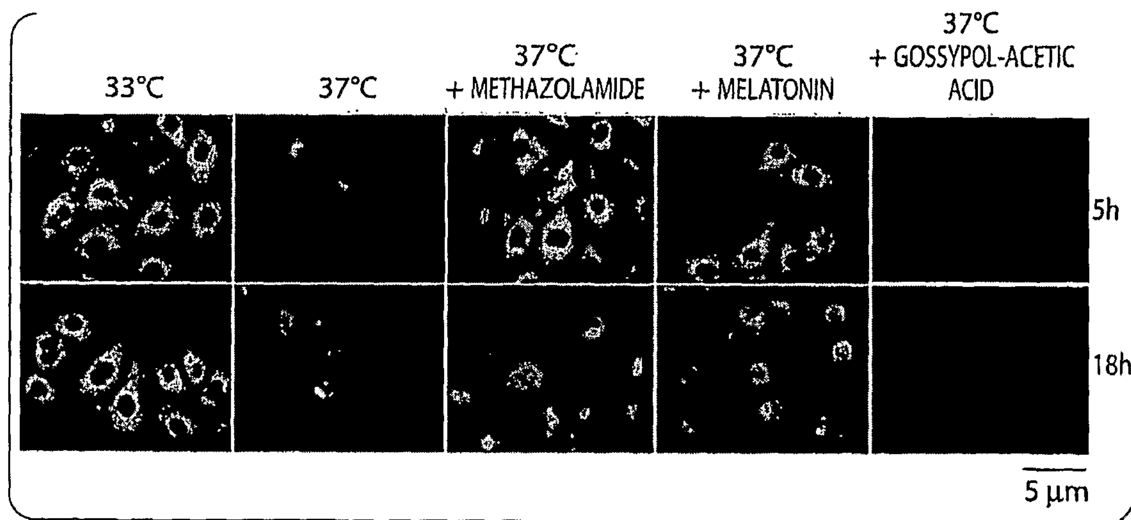


Fig. 3C

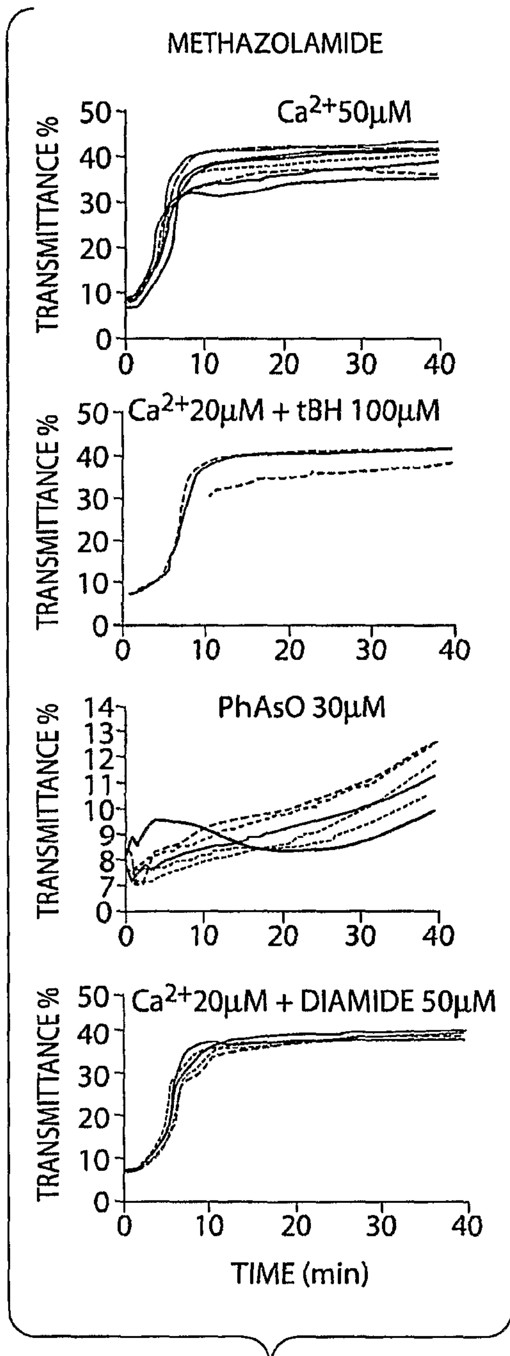


Fig. 3D

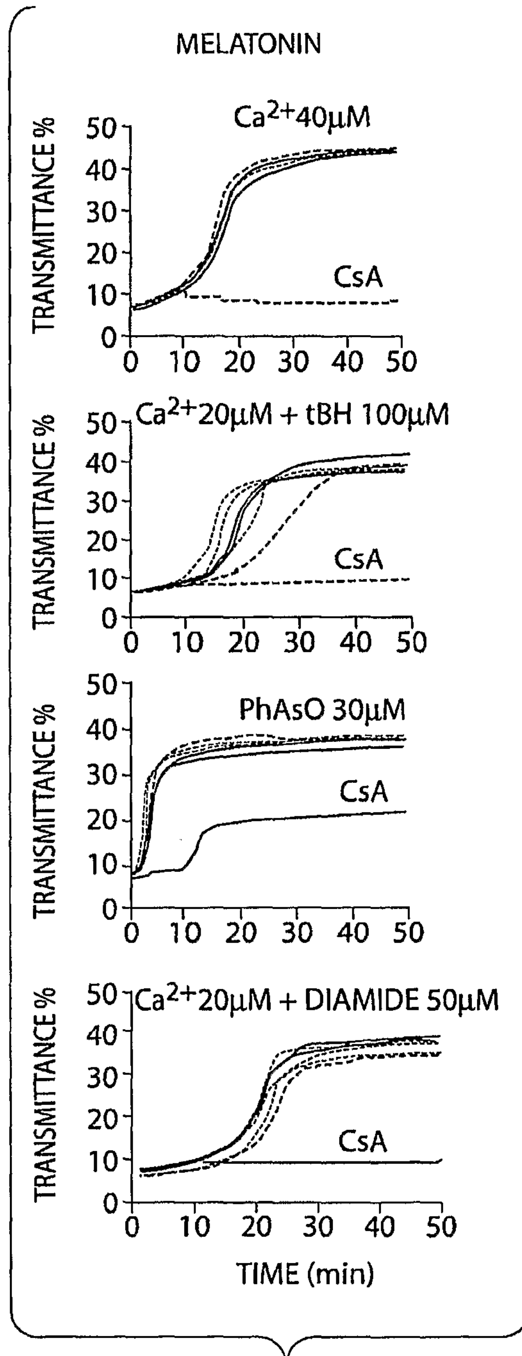


Fig. 3E

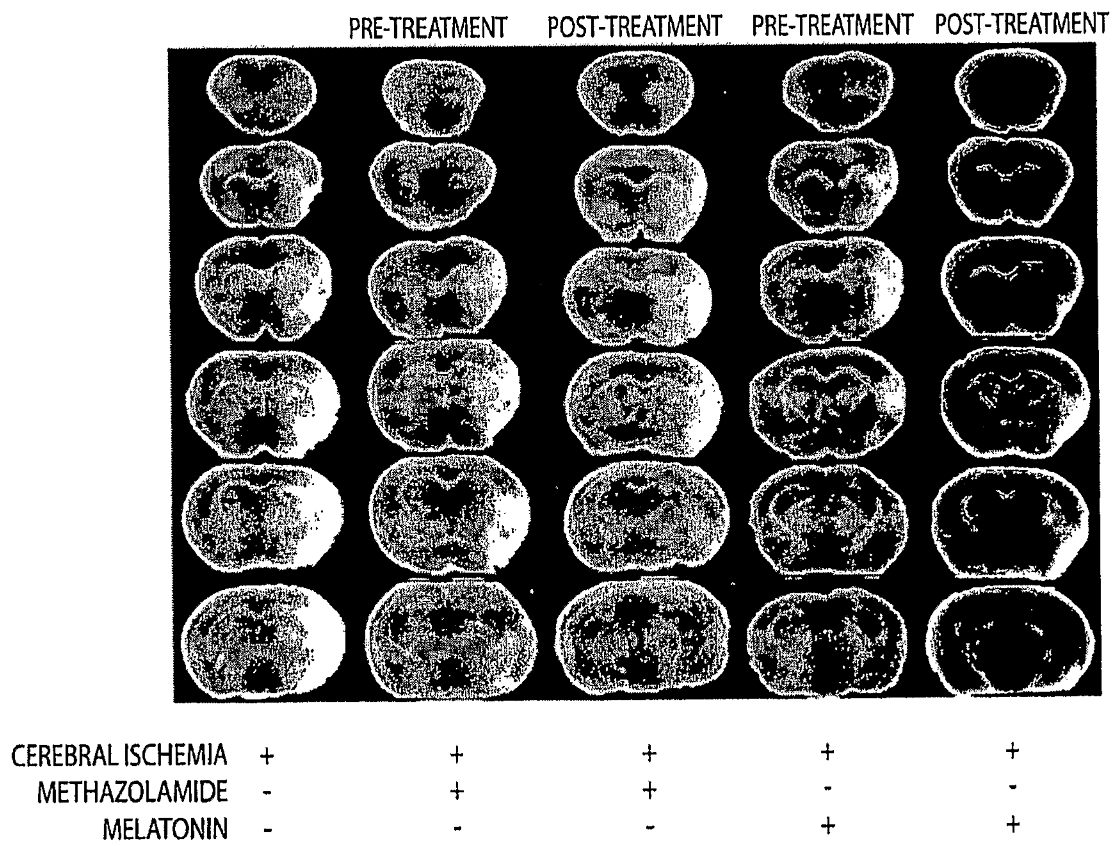


Fig. 4A

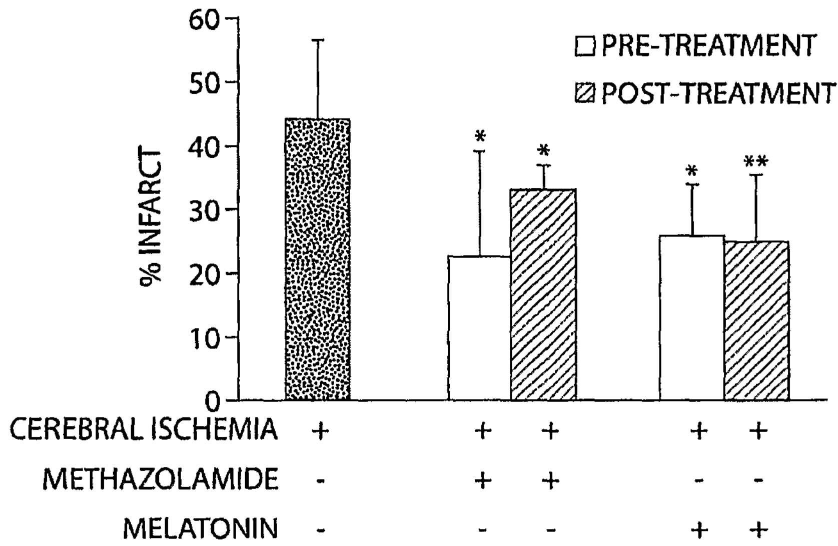


Fig. 4B

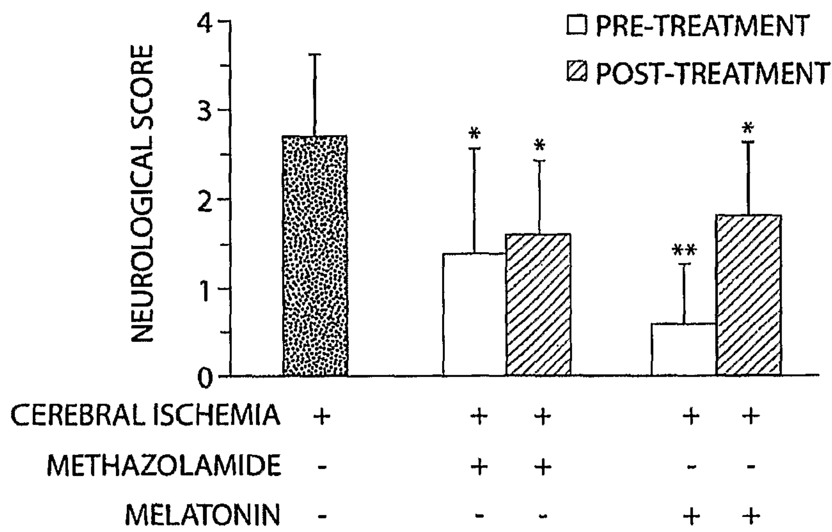


Fig. 4C

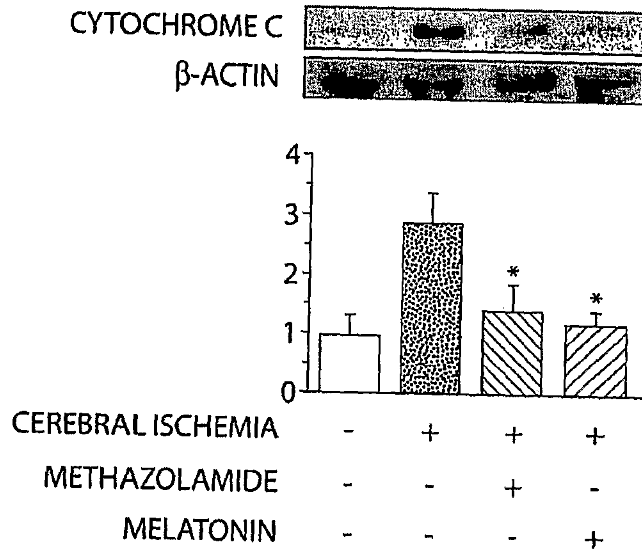


Fig. 4D

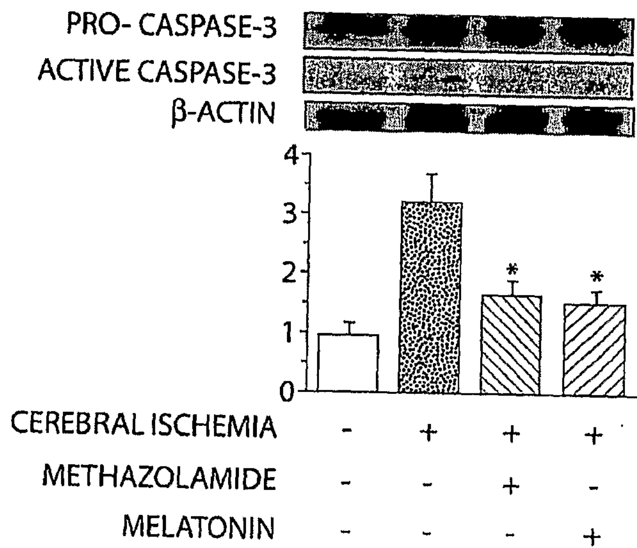
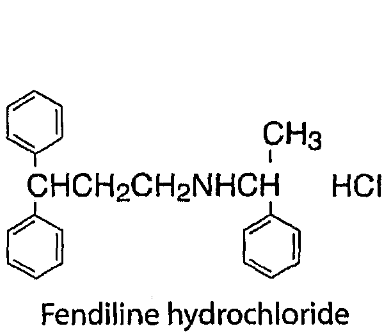
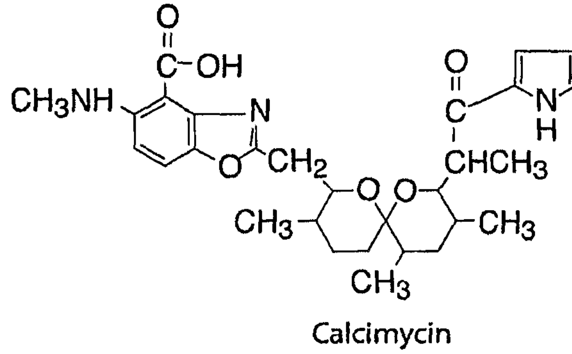


Fig. 4E

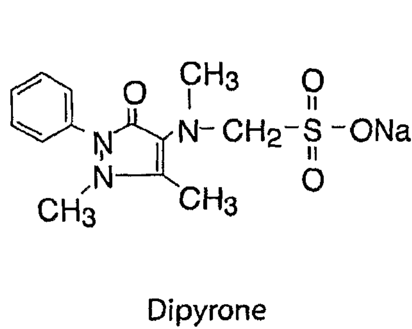
GROUP I



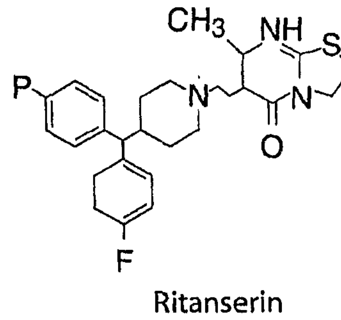
Fendiline hydrochloride



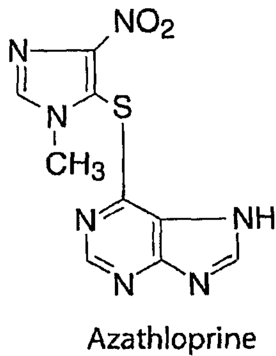
Calcimycin



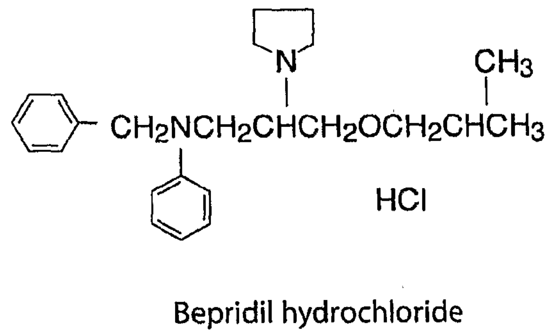
Dipyrone



Ritanserin



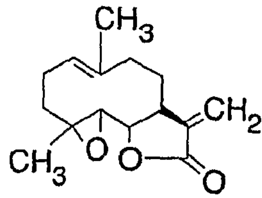
Azathloprine



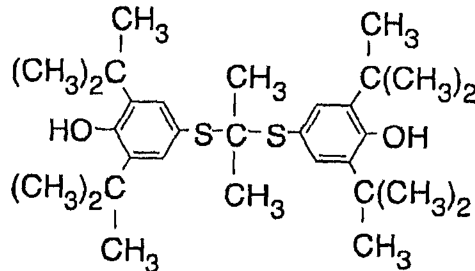
Bepridil hydrochloride

Fig. 5-1

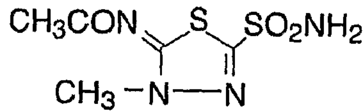
GROUP II



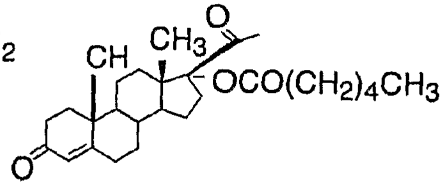
Parthenolide



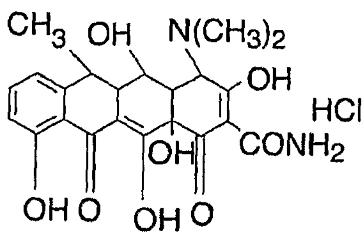
Probucol



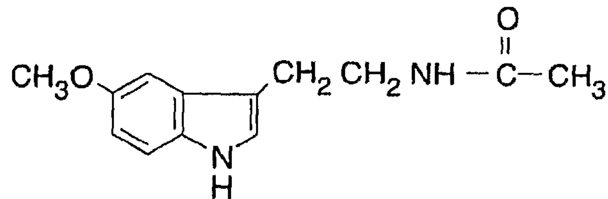
Methazolamide



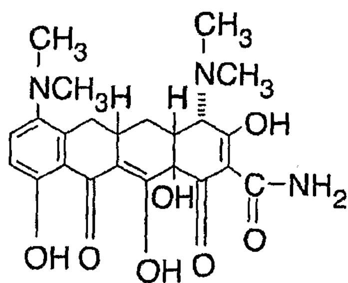
17 a-Hydroxyprogesterone caproate



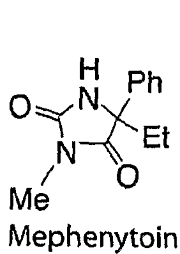
Doxycycline hydrochloride



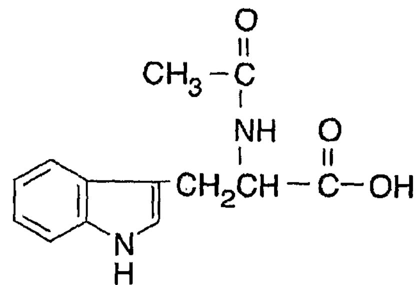
Melatonin



Minocycline hydrochloride



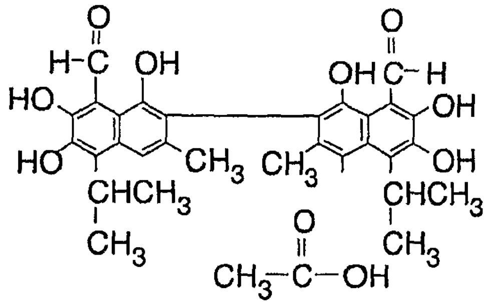
Mephenytoin



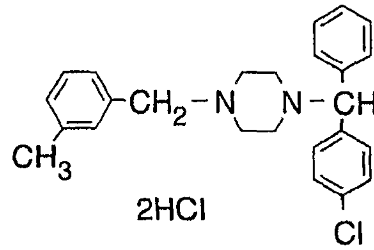
N-Acetyl-DL-lryplophan

Fig. 5-2

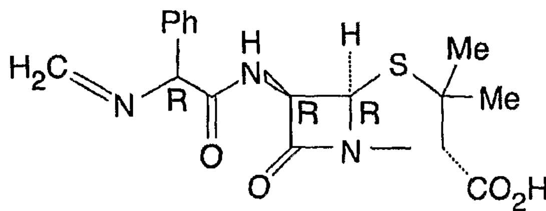
GROUP III



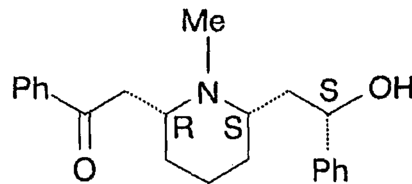
Gossypol-acetic acid



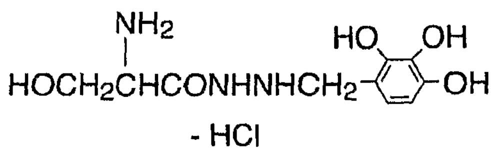
Meclizine hydrochloride



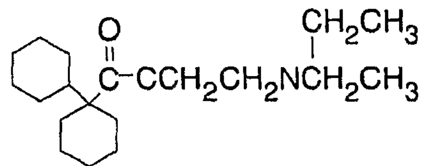
Metampicillin



Lobeline sulfate



Beserazide hydrochloride



Dicyclomine hydrochloride

Fig. 5-3

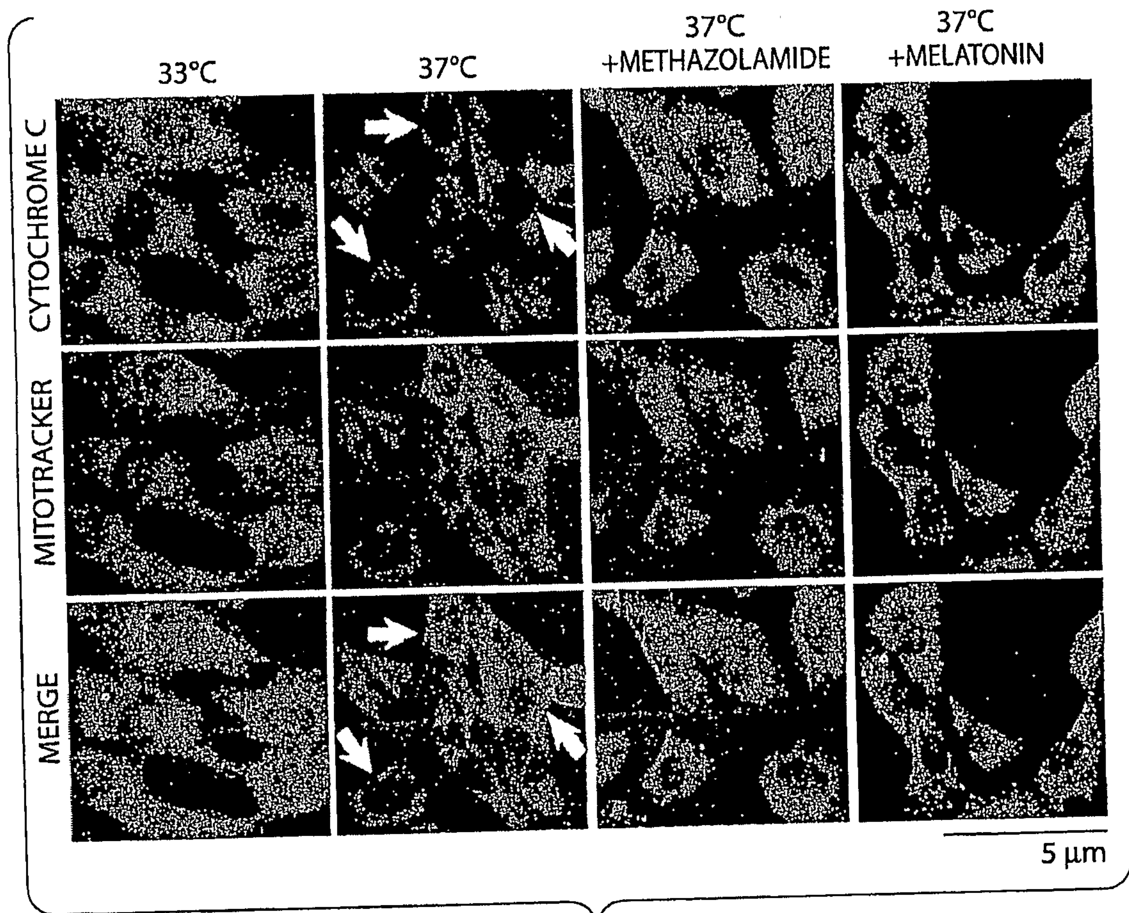


Fig. 6A

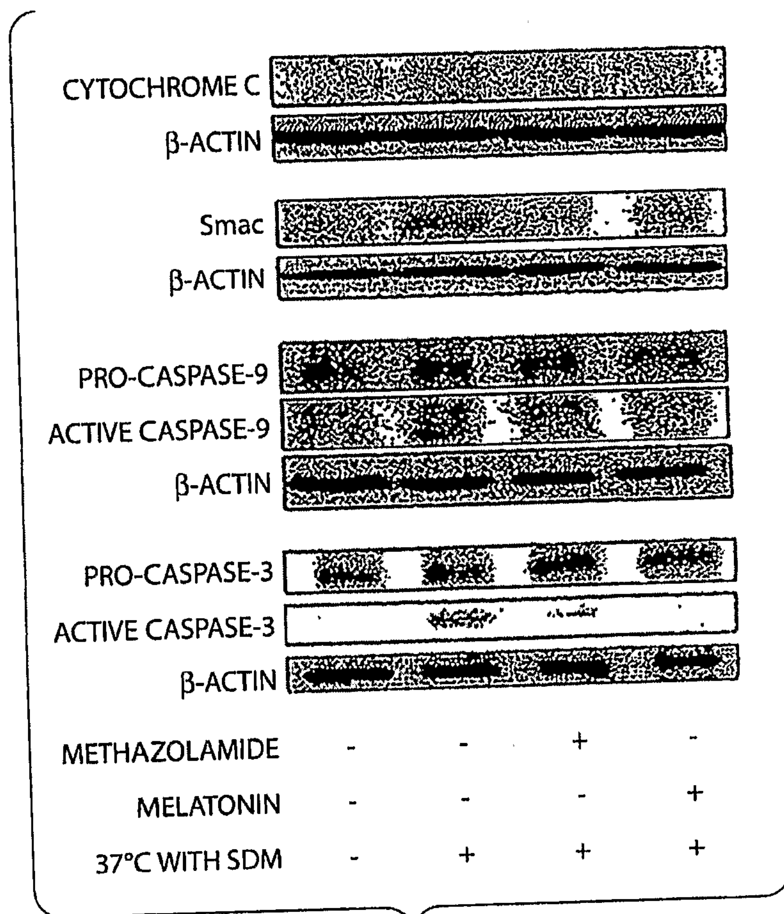


Fig. 6B

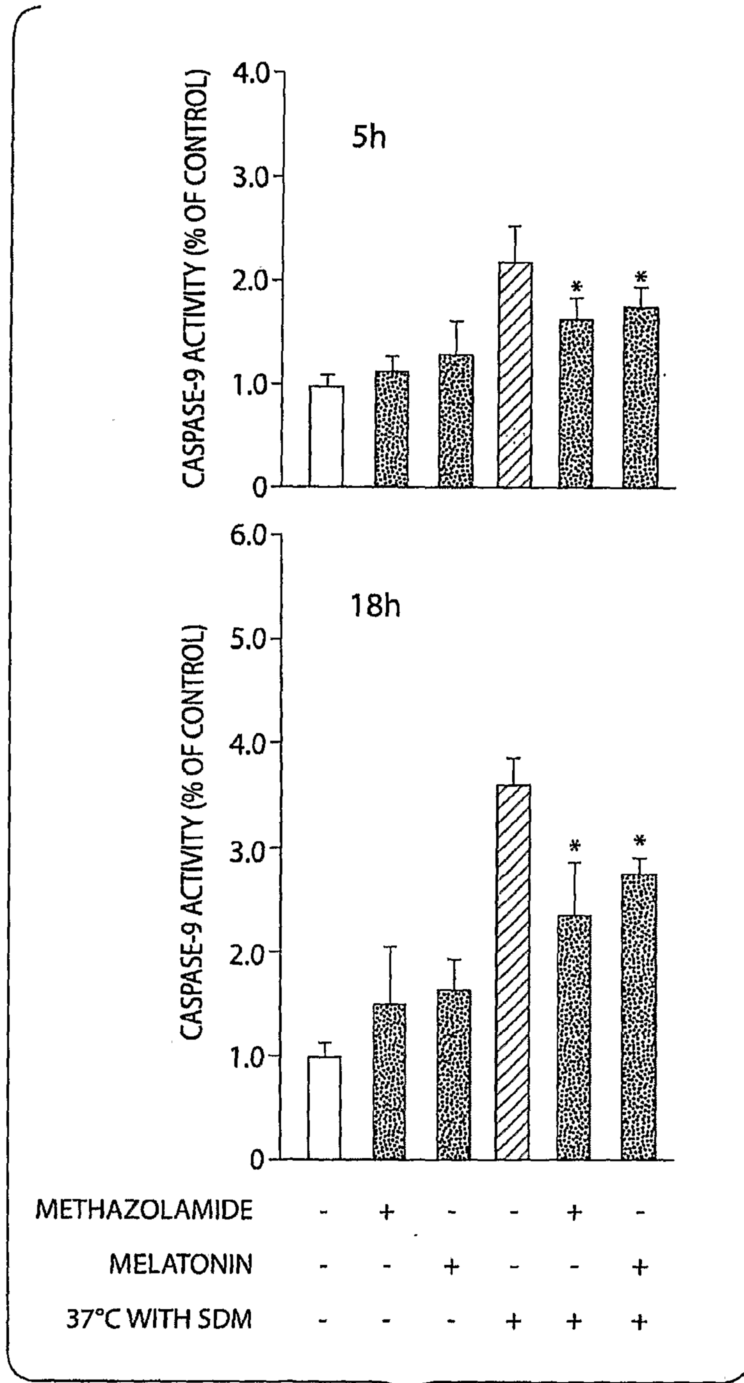


Fig. 6C

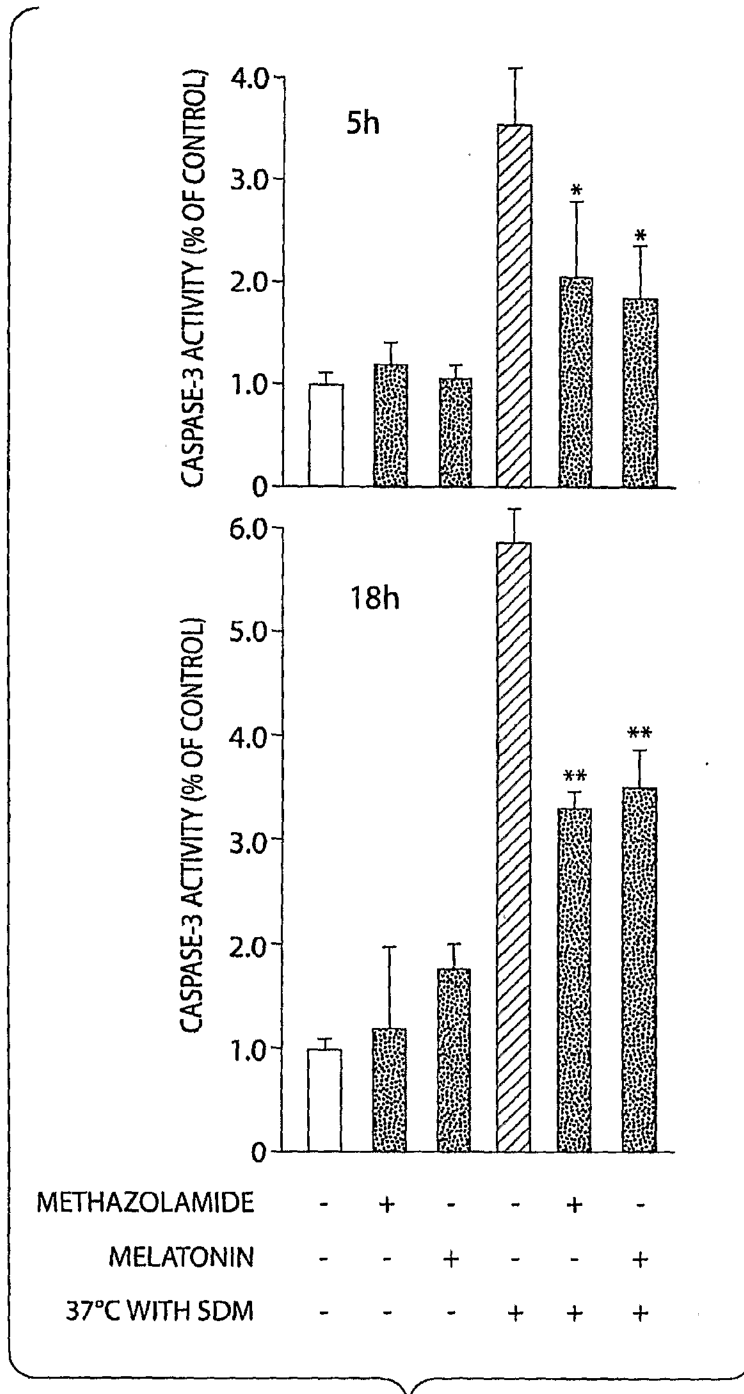


Fig. 6D

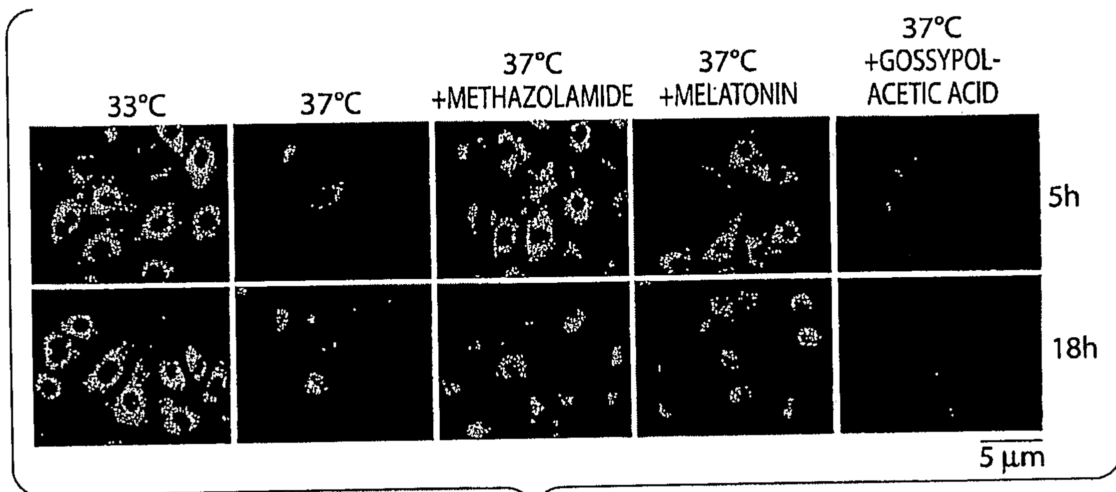


Fig. 7A

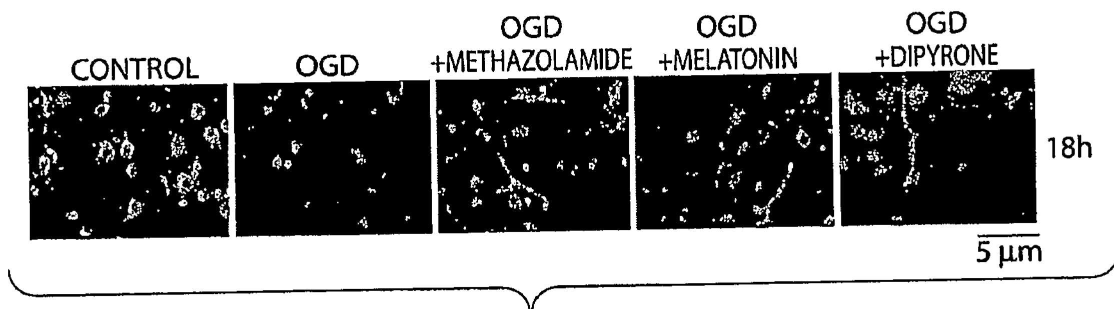


Fig. 7B

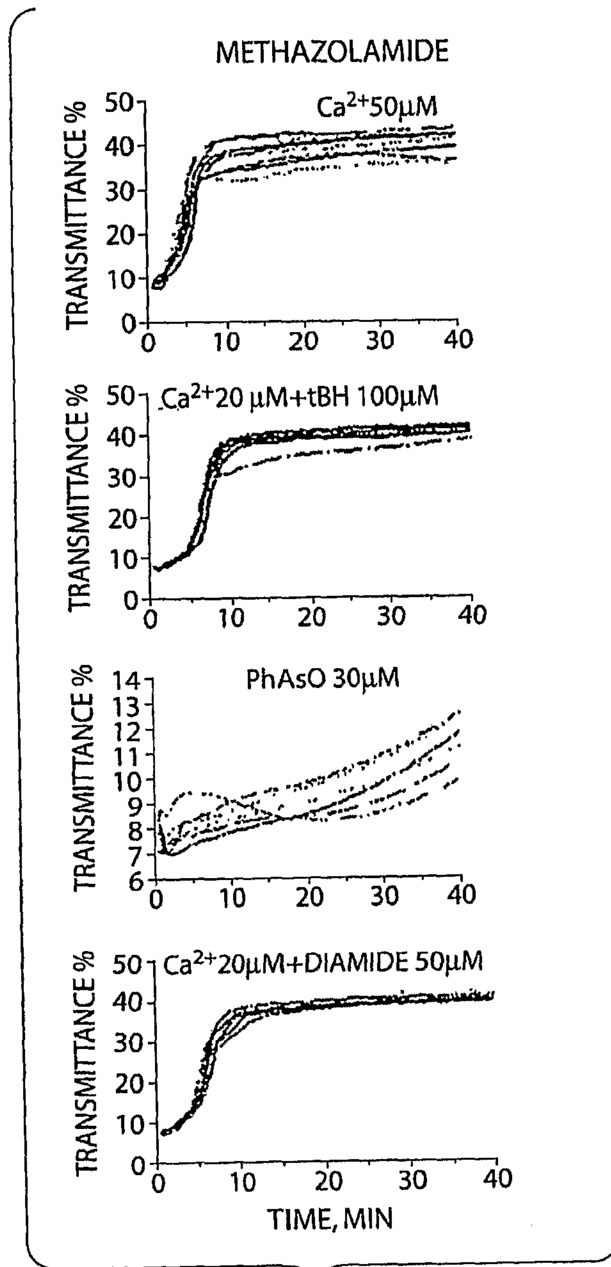


Fig. 7C

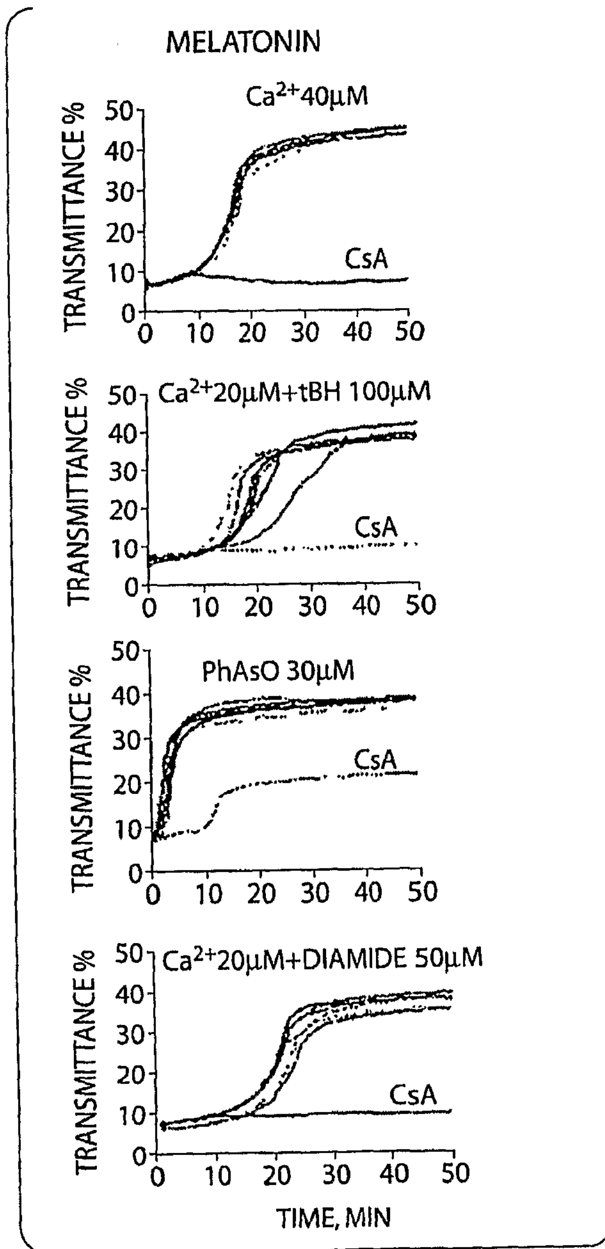


Fig. 7D

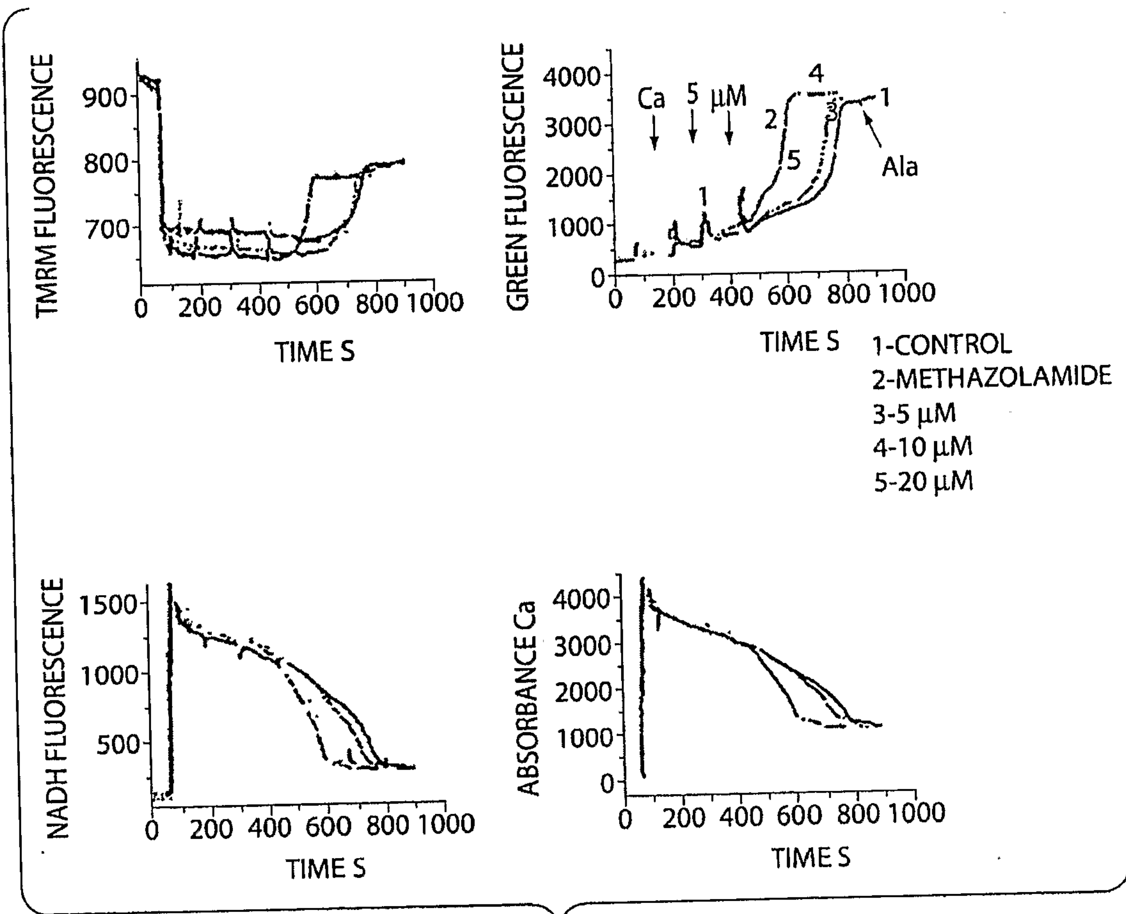


Fig. 7E

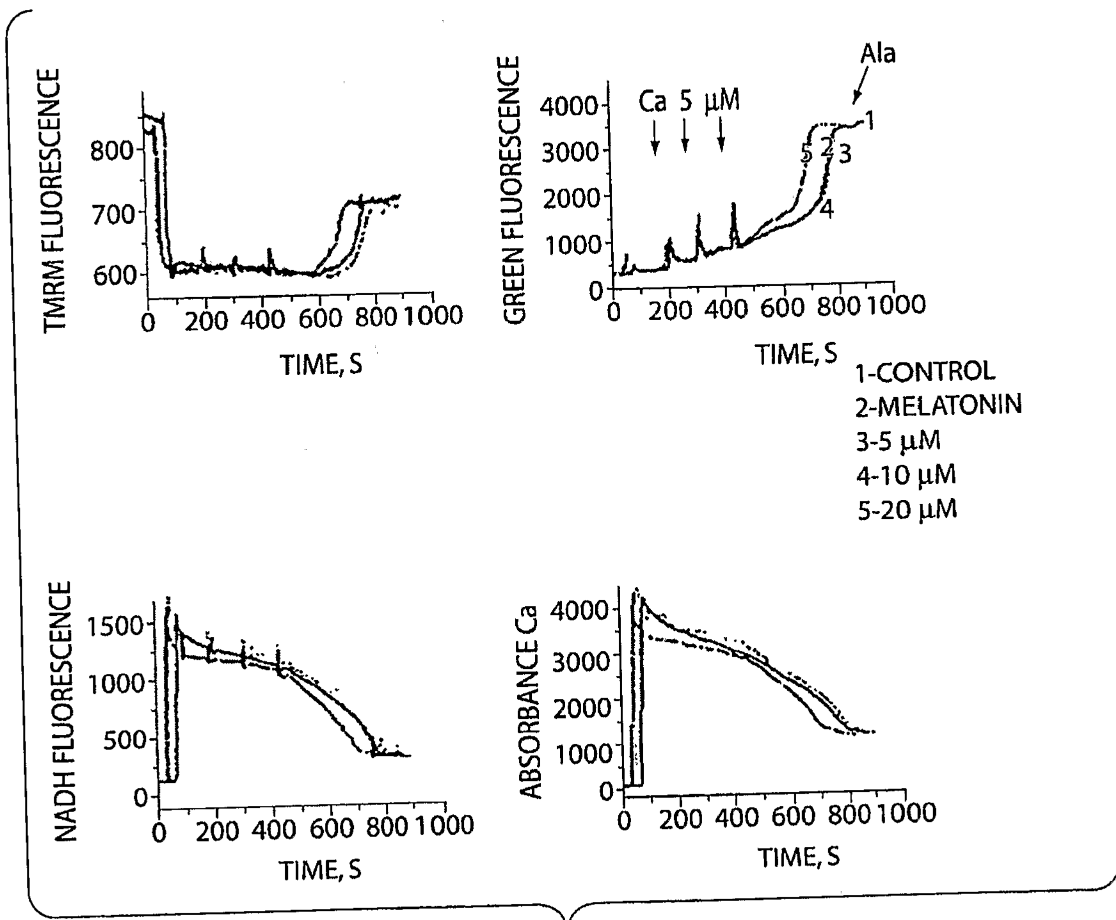


Fig. 7F

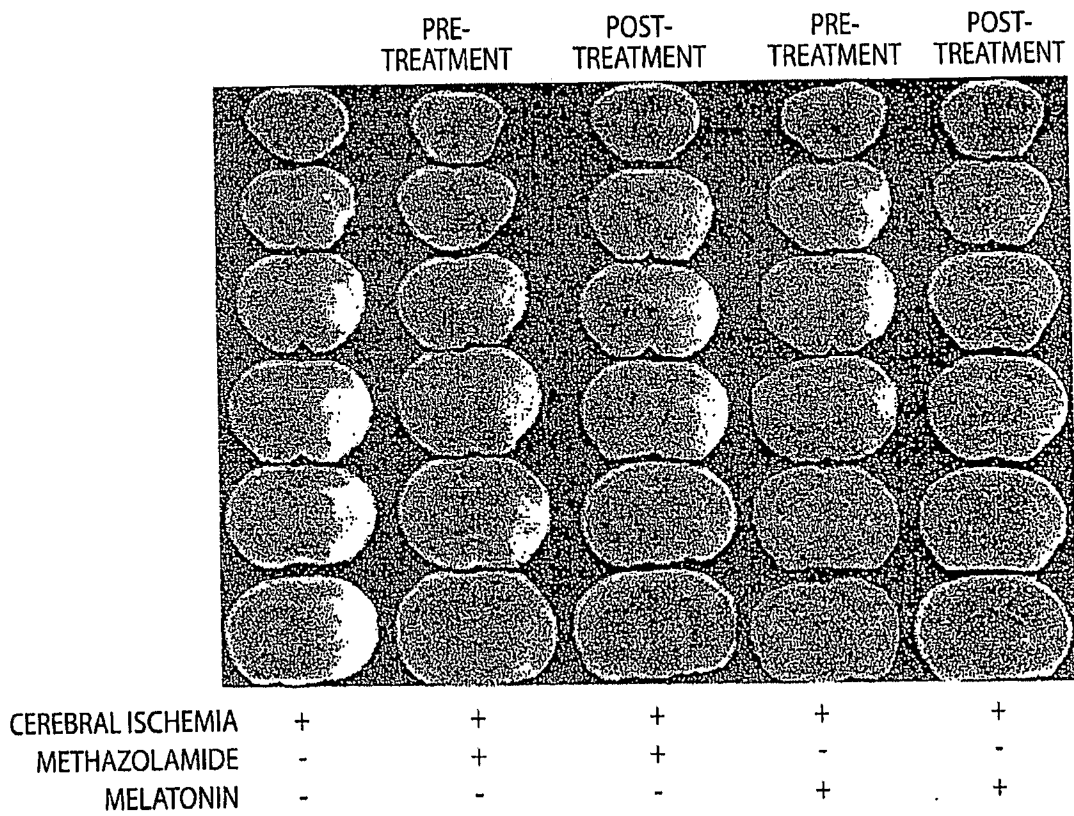


Fig. 8A

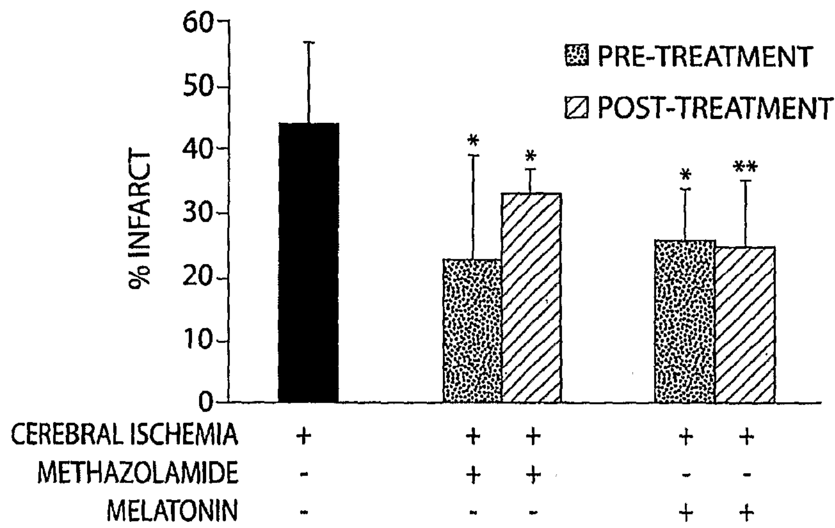


Fig. 8B

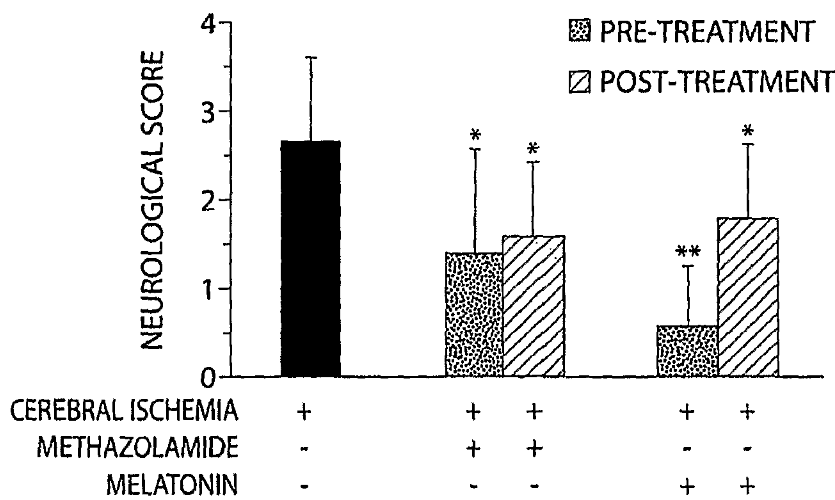


Fig. 8C

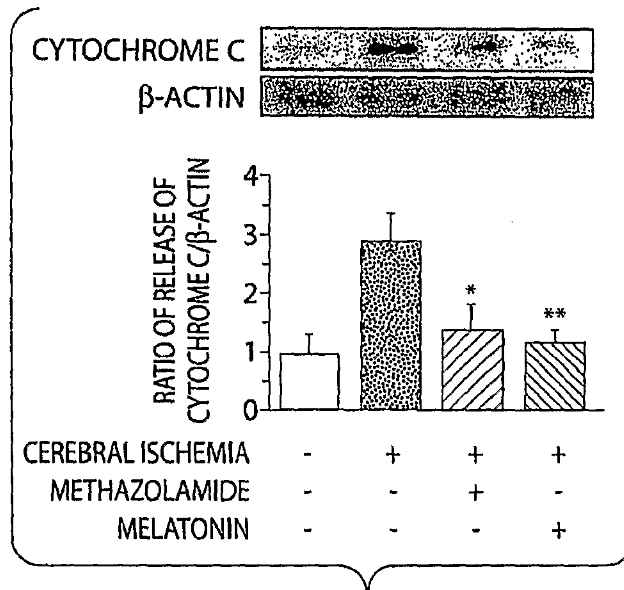


Fig. 8D

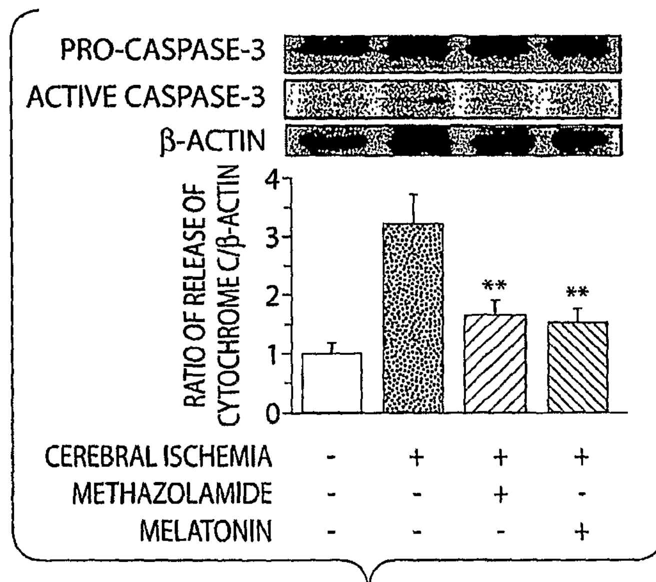


Fig. 8E

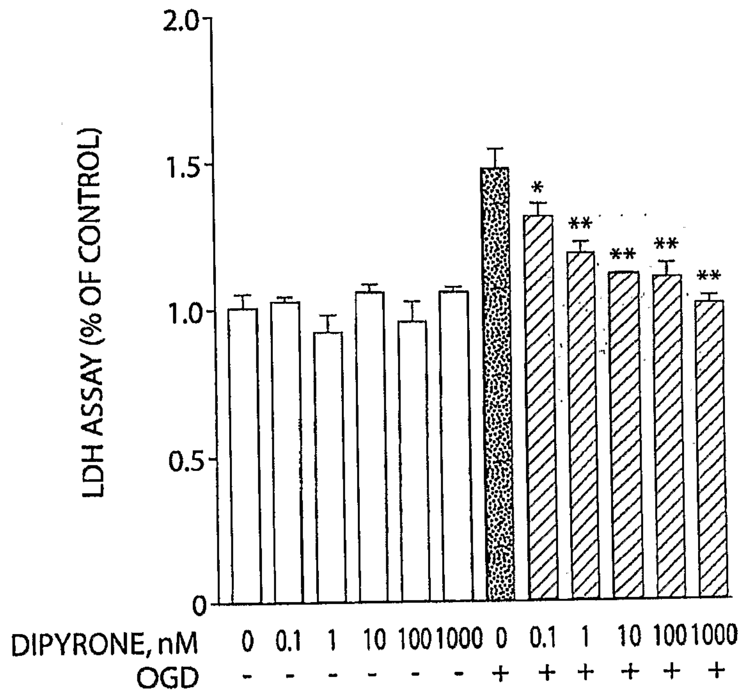


Fig. 9A

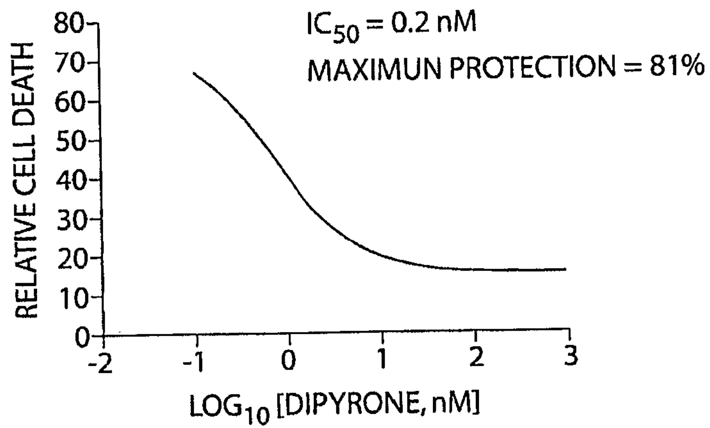


Fig. 9B

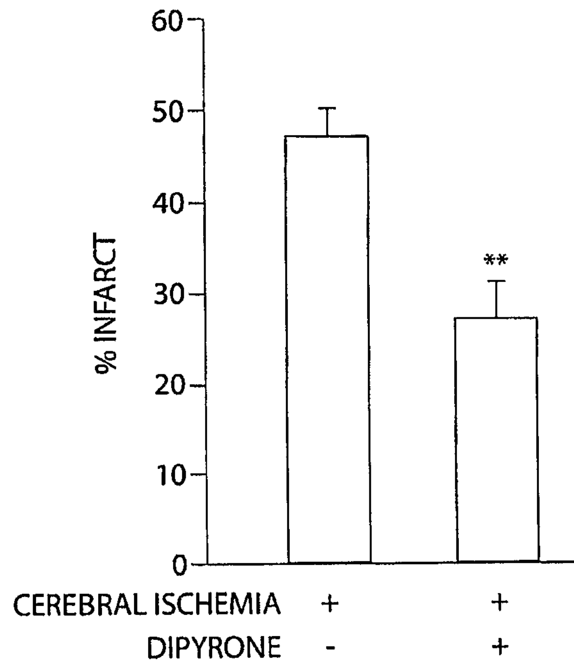


Fig. 9C

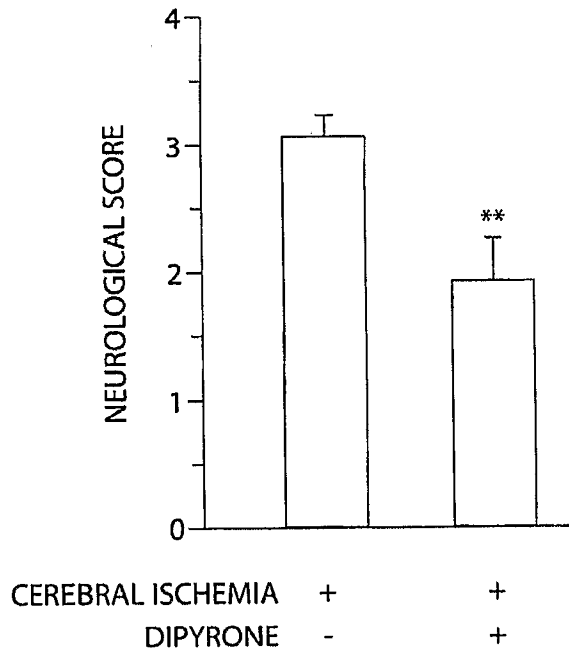


Fig. 9D

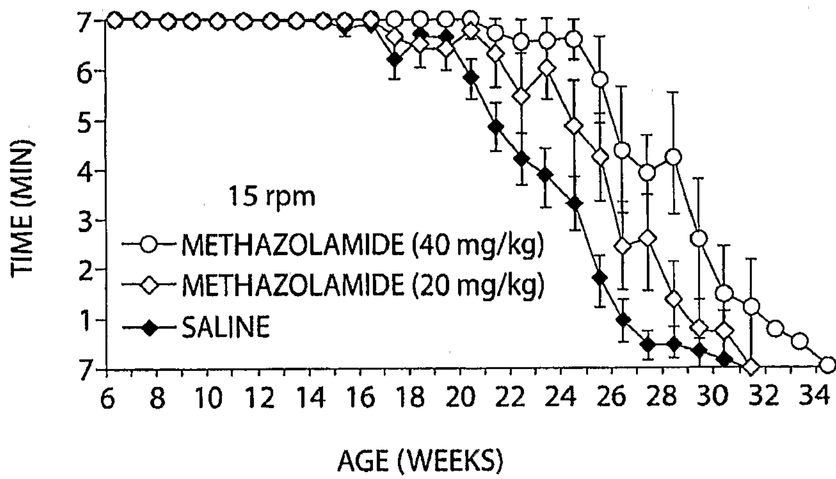


Fig. 10A

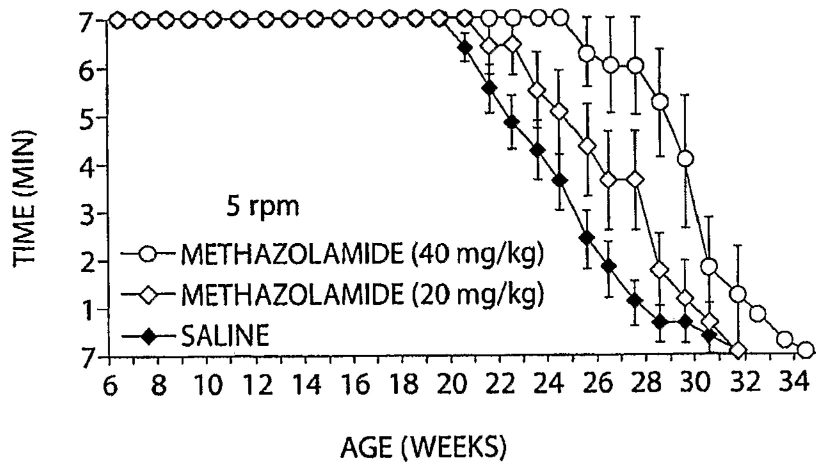


Fig. 10B

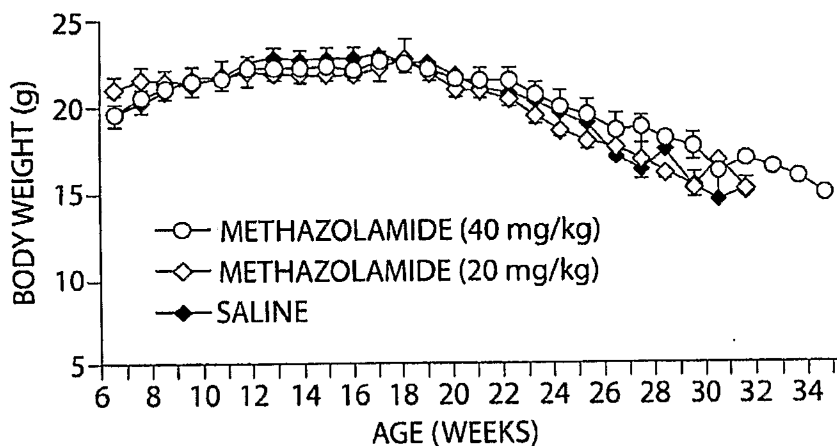


Fig. 10C

	SALINE	METHAZOLAMIDE (20 mg/ml)	METHAZOLAMIDE (40 mg/ml)
ONSET	156.7 ± 4.2	180.8 ± 6.0*	199.0 ± 6.1**
MORTALITY	169.6 ± 4.6	188.3 ± 5.8*	203.9 ± 6.9**

Fig. 10D

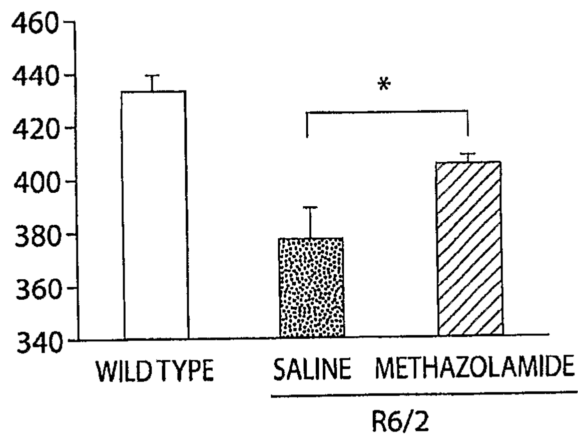


Fig. 10E

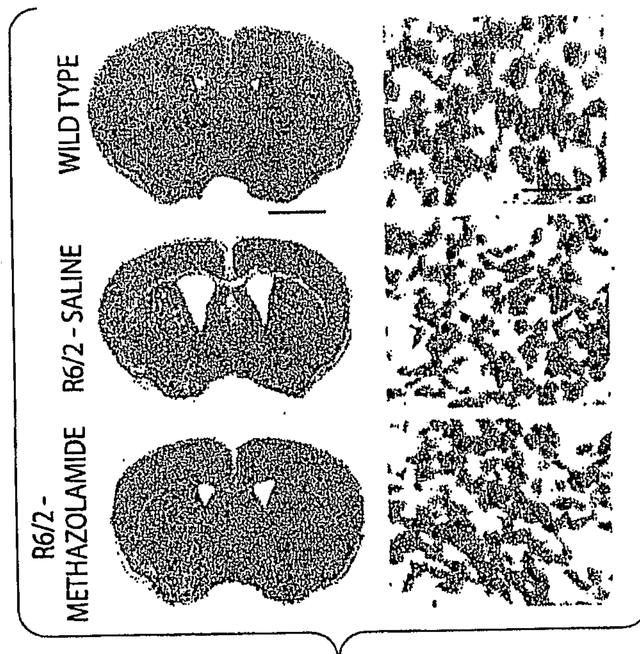


Fig. 10F

MELATONIN (30mg/kg) P<0.001*

	SALINE	MELATONIN
ONSET	157.67 ± 3.98	193.3 ± 6.36*
DURATION	14.92 ± 2.65	13.7 ± 4.5
MORTALITY	171.58 ± 4.59	207 ± 4.59*

Fig. 11

MELATONIN (10mg/kg) P<0.01*

	SALINE	MELATONIN
ONSET	155.14 ± 4.27	171 ± 3.12*
DURATION	14.14 ± 3.67	1.58 ± 0.79*
MORTALITY	169.29 ± 3.66	172.58 ± 2.74

Fig. 12

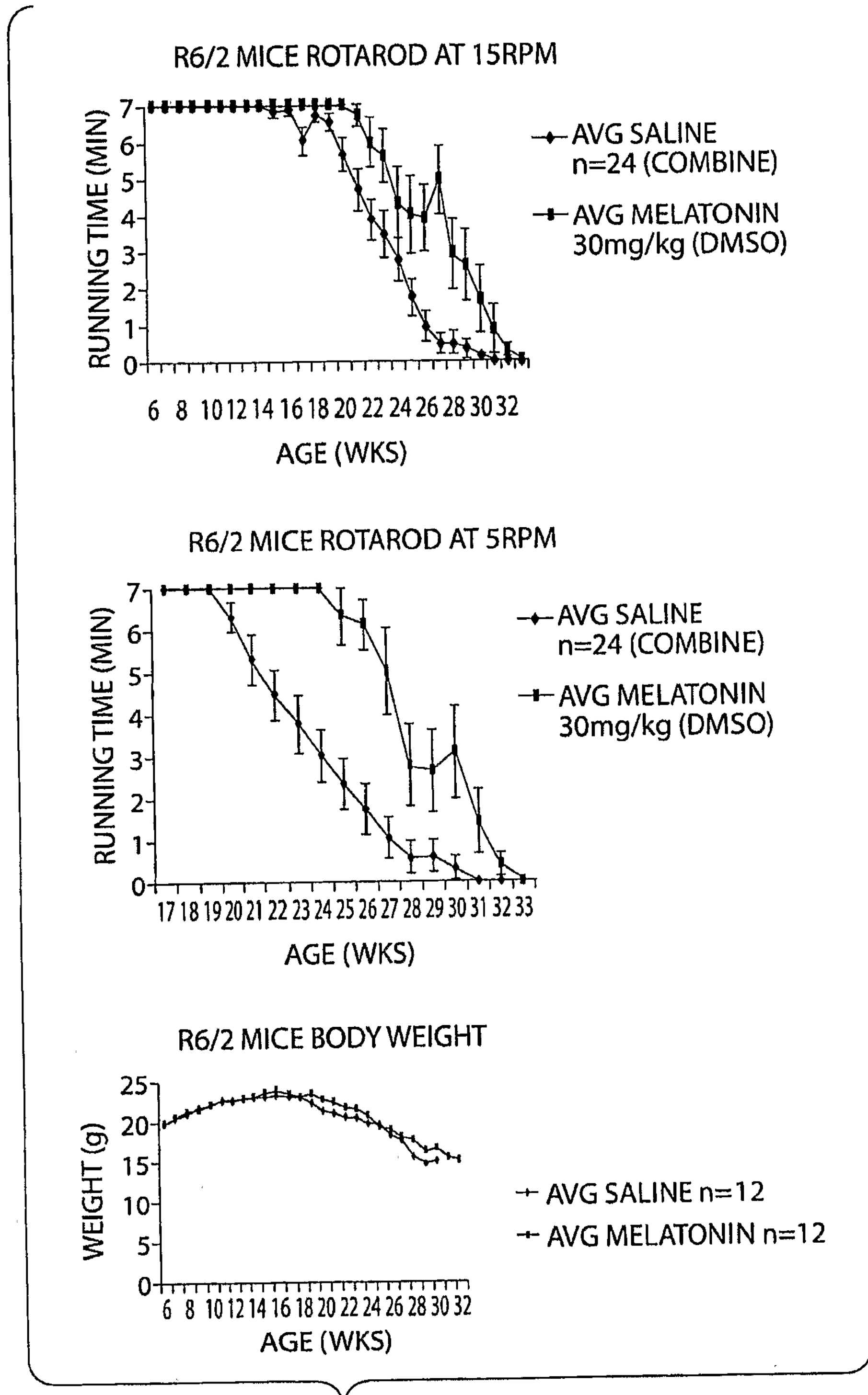


Fig. 13