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Discovery of Molecular Mechanisms of Neuroprotection Using Cell Based Bioassays and Oligonucleotide Arrays

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Running Title

Neuroprotection revealed by bioassays and microarrays

Abstract

Oxidative injury and the resulting death of neurons is a major pathological factor involved in numerous neurodegenerative diseases. However, the development of drugs that target this mechanism remains limited. The goal of this study was to test a compound library of approved Food and Drug Administration drugs against a hydrogen peroxide induced oxidant injury model in neuroblastoma cells. We identified 26 neuroprotective compounds, of which megestrol, meclizine, verapamil, methazolamide, sulindac, and retinol were examined in greater detail. Using large-scale oligonucleotide microarray analysis we identified genes modulated by these drugs that might underlie the cytoprotection. Five key genes were either uniformly upregulated or downregulated by all six drug treatments, namely tissue inhibitor of matrix metalloproteinase (TIMP1), ret-proto-oncogene, clusterin, galanin, and growth associated protein (GAP43). Exogenous addition of the neuropeptide galanin alone conferred survival to oxidant stressed cells, comparable to that seen with the drugs. Our approach, which we term "interventional profiling" represents a general and powerful strategy for identifying new bioactive agents for any biological process, as well as identifying key downstream genes and pathways that are involved.

Keywords

Oxidative stress, neuroprotection, oligonucleotide arrays, galanin

Introduction

Oxidative injury and the resulting apoptotic and necrotic death of neurons is a major pathological factor involved in numerous neurodegenerative diseases including amyotrophic lateral sclerosis (ALS), Parkinson's Disease (PD) and Alzheimer's Disease (AD) (5, 12, 21, 43). In particular, protein oxidation and the aggregation of proteins resulting from oxidative stress play important roles in the pathogenesis of these neurodegenerative diseases (9). These findings have led to the development of numerous *in vitro* and *in vivo* models of oxidative stress in neuronal systems which are commonly being used to explore the mechanistic pathways and potential therapies for neurodegeneration (1, 11, 23, 28, 33-35, 40, 50). For example, in familial ALS mutations in a gene encoding superoxide dismutase (SOD1) have been linked with the rapid progression of the disease (6, 14, 50). In PD mutations in α-synuclein and parkin are associated with early onset and rapid progression of PD and oxidative stress has been implicated (11, 21, 33, 25, 47, 62). In AD oxidative stress has also been reported to promote the accumulation of beta-amyloid (Abeta) through enhancing the amyloidogenic pathway (4, 44). However drugs that target these neurodegenerative diseases is limited. In the case of ALS there is only one therapy approved to treat ALS with many shortcomings in toxicity (32, 45, 55). The therapies available for PD are restricted to symptomatic treatment (62). Four compounds are currently approved for the use in treating AD but these compounds provide only symptomatic benefits rather than modifying the progression of the disease (26).

The drug discovery process is undergoing a revolution with the use of combinatorial chemical libraries, high throughput drug screening technologies, and DNA microarrays (3, 8, 16, 19, 60). For example, the complementary approaches of high throughput drug screening and large-scale gene expression profiling were used to screen 60 human cancer cell lines resulting in

the identification of activity profiles of numerous compounds (53, 54). This large-scale human cancer cell line study opened the door to identifying novel "gene-gene, gene-drug, and drug-drug relationships" (54, 60). In addition, numerous bioinformatics methods are currently being used to identify novel gene interactions and genes with related functions (8, 52). Although these studies did not directly assess the influence of drugs on gene expression, they served to show the power of combining large-scale drug screening with gene expression analysis.

In the present study we sought to extend this approach by testing a library of known bioactive compounds, consisting of Food and Drug Administration approved drugs, to identify those that protect human SH-SY5Y neuroblastoma cells from lethal oxidative stress. Once we identified a subset of neuroprotective drugs, we then used oligonucleotide microarrays to measure their influence on large-scale gene expression profiles and thereby elucidate the apparent mechanisms of neuroprotection that involves enhanced expression of the neuropeptide galanin in every case.

Materials and Methods

FDA2000 drug library: Our laboratory developed a drug database that contains information on US Food and Drug Administration approved drugs including drug indications, contraindications, chemical formulae, and mechanism of action. Using the Physician's Desk Reference, we identified approximately 15,000 drugs that are presently marketed in the US. Of those 1,345 were identified as unique chemical entities. We next created a drug repository of 880 compounds, and the drug collection is known as the FDA2000 drug library. The drugs/compounds were obtained from Sigma-Aldrich Chemicals (St. Louis, MO), the Brigham and Women's Hospital Pharmacy (Boston, MA) or Athena Rx Home Pharmacy (San Francisco, CA). Each drug was dissolved in water to an approximate concentration of 10 mM. 96-well daughter plates were made and plates were stored at -20^{o}C.

Cell culture: Human SH-SY5Y neuroblastoma cells were kindly provided by Dr. E. Feldman (University of Michigan, Ann Arbor, Michigan). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan UT), 2% penicillin-streptomycin and incubated at 37^{o}C in a humidified atmosphere with 5% CO_2. The cells were routinely sub-cultured using 0.05% trypsin-EDTA solution. The cells were seeded at 10^3 cells/well in 96-well plates (Corning Inc, Corning NY) and grown until each well was 75-80 % confluent.

Oxidant injury and drug screening: Our drug screening strategy was multi-staged. To take advantage of the potential pleiotrophic and latent actions of drugs in cell based bioassays, we decided to pretreat cells with the drugs for 24 hours prior to H_2O_2 exposure. Further, as many drugs can have a direct impact on H_2O_2 itself (e.g., direct antioxidants), we chose to remove the drugs prior to H_2O_2 treatment. Furthermore, to ensure that the actions of the drugs did not wear

off, we opted to perform an initial screen using a high dose of H_2O_2 for a short exposure time. Then knowing which drugs were active, we performed a secondary screen using a lower dose, longer exposure H_2O_2 protocol.

Pilot studies were performed to optimize the dose and time for exposure of cells to hydrogen peroxide. SH-SY5Y cells were incubated with various doses of hydrogen peroxide, ranging from 1-10 mM, and for different times, ranging from 4–24 hr (data not shown). An optimal dose was determined to be that which resulted in approximately 70% loss of cell viability, as this provided maximal signal-noise in identifying drugs that rescued cells from death. For the initial high dose screen, cells were exposed to 6 mM hydrogen peroxide for 4 hr. In the secondary low dose H_2O_2 screen to confirm drug efficacy and optimize drug doses, cells were exposed to 100 μM hydrogen peroxide for 24 hr. In each 96-well plate, 8-wells were used as controls in which no hydrogen peroxide was added.

Drugs were prepared by prediluting them to a concentration of 10-100 μM in DMEM containing 10% fetal bovine serum. Cell culture medium was removed from the cells in the 96-well plates and replaced with the fresh medium containing the drug (10-100 μM). There was only one drug per well. Cells were incubated with the drug for 24 hr at 37°C. Then cell culture medium and drugs were removed and the cells washed once with D-PBS. Those drugs found to be protective in this first screen were then investigated in the secondary low dose hydrogen peroxide screen. Drug dose-response experiments were performed using one protective drug from six different therapeutic classes to identify a peak effective dose. The SH-SY5Y cells were incubated with each drug (10-100 μM) for 24 hr, drugs were removed, and cells were exposed to 100 μM hydrogen peroxide for 24 hr at 37°C.

To measure cell viability, the cell culture medium containing H_2O_2 was removed and

replaced with D-PBS containing 10 μM calcein-AM (Molecular Probes, Eugene OR) and cells were then incubated at 25°C for 30 min. Fluorescence was measured using a Victor^2 Multilabel fluorescence plate reader (PerkinElmer Life Sciences, Boston MA).

RNA isolation and oligonucleotide arrays: Cells were incubated for 24 hours with drugs at peak effective concentrations as follows: 30 μM megestrol, 60 μM meclizine, 30 μM verapamil, 100 μM methazolamide, 10 μM sulindac, and 10 μM retinol; there was no exposure to H_2O_2. Total RNA was isolated using Trizol (Gibco BRL Life Technologies, Rockville MD) and RNA integrity was tested by visualization of 18S and 28S bands. Total RNA (5-7.9 μg) was used for in vitro transcription and labeled with biotin following procedures described previously (31, 39, 61). Following verification of cRNA quality on Test2 GeneChips, Affymetrix HG-U95A GeneChip probe arrays were used to determine mRNA expression levels.

Data analysis and informatics: Drug screening data were analyzed using Microsoft Excel to assess standard statistical parameters. The oligonucleotide arrays were analyzed using MicroArray Suite 4.0 software from Affymetrix, with a "target intensity" (mean expression level) of 100. Significant changes in gene expression were identified by an average 2-fold or greater change across different protective drug treatments with $P<0.003$ calculated using a single sample, two-tailed *t*-test applied to the logarithms of the ratio of the drug treated gene expression levels to the control levels. This analysis was repeated for two sets of microarrays for the control and drug treatments (with n=5 and n=6). A small set of genes was identified that satisfied these requirements in both of the replicate experiments. These stringent criteria were designed to minimize the number of false positives and to generate a short list of informative genes.

Results

For our primary drug screen SH-SY5Y cells were incubated with each of the 880 drugs in the library at concentrations of 10 and 100 μM for 24 hr. The drugs were then removed, a lethal dose of hydrogen peroxide was added for 4 hours, and cell viability assayed. As shown in Table 1, we identified 26 compounds that provided 50-100% cytoprotection of SH-SY5Y cells, as compared to control cell cultures in which no drugs were added. Interestingly these compounds fell into multiple therapeutic classes suggesting multiple mechanisms may be involved. The drugs were grouped into therapeutic classes and one drug was chosen from the different classes for further testing, namely megestrol, meclizine, verapamil, methazolamide, sulindac, and retinol. To determine the optimal protective doses, the six protective drugs were added at concentrations ranging from 1-300 μM for 24 hr and assayed for their ability to abrogate high dose H_2O_2 (6 mM) mediated cell death (Table 2). Each drug showed a classic dose-response. Increasing doses provided greater cytoprotection until an optimal protective dose was attained, after which additional drug caused toxicity.

Having identified a subset of six neuroprotective compounds, we next evaluated their efficacy in a low dose H_2O_2 toxicity assay. The optimal doses of each compound were added to SH-SY5Y cells for 24 hours, drugs were removed, and cell survival was assessed following a 100 μM H_2O_2 exposure for 24 hours in the absence of the drug. As shown in Figure 1, this prolonged application of H_2O_2 resulted in 66 ± 9 % cell death in the absence of any drug. All six drugs were cytoprotective, with all compounds except sulindac promoting survival that was no different from control cells. Sulindac showed partial protection with 59 ± 9 % viability observed.

To check that the measured levels of neuroprotection were not the result of enhanced proliferation induced by the drugs we used Hoechst 33258 pentahydrate nucleic acid stain and

calcein-AM. After 24 hours of exposure to the drugs the normalized cell numbers averaged 109 ± 8% for Hoechst stain and 85 ± 9% for calcein-AM compared to untreated controls, 100 ± 11% Hoechst stain and 100 ± 16% calcein-AM.

Having identified a highly diverse group of neuroprotective pharmaceutical compounds whose known mechanisms of action are highly unrelated to each other or to cell survival, we sought to discover a biological process that would interconnect them. Using high-density oligonucleotide microarrays, RNA expression levels of approximately 12,000 genes were measured in SH-SY5Y cells incubated for 24 hr with optimal doses of megestrol, meclizine, verapamil, methazolamide, sulindac, and retinol. RNA was also isolated from vehicle treated control cells. Both the drug treatments and the control were repeated on two separate occasions. These replicate experiments provided two sets of microarray measurements of the gene expression changes due to the protective drugs (however one of the microarrays for megestrol was discarded from the data set for due to poor chip quality). These two sets of measurements of gene expression changes induced by the drugs were then analyzed using single sample, two-tailed Student's *t*-tests to determine a small subset of genes with the most significant changes in expression. As shown in Figure 2, data analysis identified a small subset of genes that were differentially expressed in response to all six drugs. These included increased expression of tissue inhibitor of matrix metalloproteinase, TIMP1 (D11139), clusterin (M25915), galanin (M77140), and GAP43 (M25667) and decreased expression of ret-proto-oncogene (HG4677-HT5102). Quantitative RT-PCR was used to confirm the increased expression for two of these genes, clusterin and galanin, for every drug treatment. Although the five genes do not appear to be involved in any common pathways, they all play critical roles in the normal physiological functions of neural cells.

Galanin is a known to be a secreted neuropeptide that is involved in the neurotransmission of neural cells (49). Being a secreted peptide, we reasoned that galanin may also act as an autocrine cytoprotective agent. Thus, we incubated SH-SY5Y cells with exogenous galanin for 24 hr to assess its ability to confer cell survival with subsequent exposure to 100 μM H_2O_2 for 24 hr. As shown in Figure 3, galanin alone blocked oxidant-induced cell death of the SH-SY5Y cells. We verified that the galanin alone did not enhance cell proliferation as determined using Hoechst stain (110 ± 25 vs 100 ± 18% control). Collectively, these findings suggest that the drugs that provide protection to SH-SY5Y cells were influencing the transcription of a small set of genes, and that in particular that galanin plays a key role in the neurodegenerative pathways of oxidant-induced neural injury.

Discussion

In the present study we showed that using a cell based bioassay it is possible to identify existing pharmaceuticals that are neuroprotective in a model of oxidant injury. Furthermore, by directly evaluating the effects of several of these neuroprotective agents on mRNA expression, we identified five differentially expressed genes, including galanin, which appear to be associated with cytoprotection. Exogenous addition of galanin alone recapitulated the drug-mediated neuroprotection. This approach of using drug screening followed by global analysis of gene expression represents a powerful paradigm for biological and drug discovery that could be used in many systems.

Our cell bioassay involved the use of human SH-SY5Y neuroblastoma cells which are derived from the sympathetic nervous system and possess many properties of mature sympathetic neurons. Using the well established H_2O_2 oxidant injury model, we implemented a

two step drug screening process. First using a high dose, acute cellular H_2O_2 exposure to identify candidate neuroprotective compounds and then confirming the efficacy of these agents using a lower dose and more chronic H_2O_2 exposure protocol. The drug library was comprised of 880 known pharmaceuticals. For both the acute and chronic protocols cells were exposed to the drugs for 24 hours prior to H_2O_2 exposure, enabling the compounds to directly or indirectly modulate many processes within the cells. Moreover, it is more reflective of an *in vivo* setting where drugs are taken chronically, giving cells an opportunity to manifest both primary and secondary responses.

Overall, we identified 26 drugs that provide neuroprotection, representing more than six different therapeutic classes. This relatively high "hit rate" of 3% was attained without optimizing doses or formulations in the initial screen, underscoring the mulitipotent activities of existing pharmaceuticals. Additional neuroprotective compounds may have been missed in our primary screen since they were not all tested under optimal conditions.

Our FDA2000 drug library is composed of a non-redundant set of FDA approved drugs that are presently marketed in the US. Most of these compounds have never been implicated in neuroprotection, for either their prescribed indication or known mechanisms of action. However, due to the cross reactivity of many of these drugs with other possible targets or their actions on non-target neuronal cells, a number of compounds emerged as having unanticipated actions to prevent oxidative damage in neuronal cells. This observation suggests that through the interconnected network of biological pathways and processes, drugs of highly unrelated structures and actions can have similar effects.

The six drugs examined in detail, namely sulindac, retinol, verapamil, megesterol, meclizine, and methazolamide, are highly unrelated with regard to their known targets and

primary actions. Of these, prior studies suggested sulindac, retinol, and verapmil could be cytoprotective during oxidant injury. Sulindac is a nonsteroidal anti-inflammatory drug used for the treatment of inflammatory diseases, and rheumatoid arthritis. It inhibits prostaglandin synthesis by decreasing the activity of cyclooxygenase. Sulindac has been shown to scavenge oxidant products of prostaglandin cyclooxygenase/peroxides (51). Moreover, epidemiological studies have revealed a reduction in the prevalence of AD among people taking nonsteroidal anti-inflammatory compounds (58), though sulindac has not yet been specifically implicated.

Retinol (vitamin A or all-trans-retinoic acid) is used a topical treatment of acne. Retinoic acids act upon various biological process including cell proliferation, differentiation, and cellular morphogenesis (36). Human trials of retinol revealed a reduction in reactive oxygen metabolites such as those produced by endogenous cellular hydrogen peroxide (13). Pretreatment of mesangial cells with retinol blocked morphological and biochemical markers of apoptosis typically induced by H_2O_2 (46). The anti-apoptotic effect of retinol against H_2O_2 was also observed in fibroblasts (46). This anti-apoptotic pathway was shown to act through the dual suppression of the cell death pathway mediated by c-Jun N-terminal kinase (c-Jun), and activator protein 1 (AP-1). The trans-retinoic acid anti-apoptotic pathway acts on both nuclear receptor-dependent and –independent mechanisms (36).

Verapamil belongs to the antianginal and antihypertensive therapeutic class of compounds, and is specifically an antiarrhythmic class IV drug. Verapamil inhibits calcium ions from entering the slow channels, and select voltage-sensitive areas of the vascular smooth muscle. Verapamil has possible neuroprotective effects on normal neurons exposed to high concentrations of ethanol, and it has been suggested that verapamil should be evaluated as a drug for treatment of alcohol-induced brain damage, and neurodegenerative disorders (37).

Megesterol, meclizine, and methazolamide, as well as many compounds identified in the primary screen (Table 1), are not known to be neuroprotective or cytoprotective during oxidant stress. Megestrol is an antineoplastic agent that is a synthetic analog of progesterone (10). The chemical structure of megestrol is similar to that of norethindrone and flunisolide which were also found to be protective to a lesser extent in the primary hydrogen peroxide screen. Meclizine is an antimetic and an antihistamine H_1 blocker used for the prevention and treatment of motion sickness and the management of vertigo with diseases affecting the vestibular system. Meclizine has central anticholinergic actions by blocking the chemoreceptor trigger zones (48). Methazolamide is a diuretic drug, and acts as a noncompetitive inhibitor of carbonic anhydrase (38).

To investigate potential biological actions of the protective drugs, we evaluated their effects on mRNA expression 24 hours after drug exposure using large-scale oligonucleotide microarray analysis (Fig 2). A set of five genes, TIMP1, ret-oncogene, clusterin, galanin, and GAP43, were all identified as differentially expressed in response to all six drugs (Fig 2). Their encoded proteins all play critical roles in the normal physiological functions of neural cells and several have been implicated in cell survival.

Tissue inhibitor of matrix metalloproteinase (TIMP1) was upregulated and is a member of a family of TIMP genes involved in cell proliferation and cell survival. TIMP1 may specifically inhibit apoptosis (18, 27) and can confer resistance to oxidative stress (22). Interestingly, the inhibition of matrix metalloproteinase showed a significant decrease in liver ischemia/reperfusion injury as assessed by histological and serum hepatic levels, and has been proposed to have clinical relevance in liver-associated ischemic disease (15). H_2O_2 was reported to be an important intermediate in the downstream signaling pathway leading to the induction of

an increased steady state of matrix metalloproteinase-1 mRNA levels (7). These findings provide supporting evidence for our experiments in which TIMP1 was upregulated by the protective compounds, and prevented oxidant induced injury.

Clusterin (Apolipoprotein J) was significantly upregulated in all the drug treatments as compared to the control cells. Clusterin is an 80 kDa glycoprotein that has been implicated in cytoprotection of fibroblasts and is induced by numerous cellular stresses. Over-expression of clusterin may be associated with cell survival after oxidative injury (2, 17, 57). Clusterin has been suggested to protect cells against apoptotic cell death and neurodegeneration (59). Interestingly, in AD lower cellular expression levels of the clusterin protein, was suggested to be associated with neuronal degeneration and death (20). Using an antisense approach, suppression of clusterin mRNA and protein expression made cells more sensitive to apoptotic cell death induced by heat shock or H_2O_2 induced oxidant stress (57). These studies indicate that clusterin confers cellular protection against heat shock and oxidative stress (57).

RET was decreased more than two-fold in all the drug treatments. The ret proto-oncogene encodes a cell membrane tyrosine-kinase receptor protein whose ligands belong to the glial cell line-derived neurotrophic factor family (56). Its role in cytoprotection during oxidant stress still needs to be elucidated.

Galanin expression was enhanced by all six drugs. This 29 amino acid secreted neuropeptide colocalizes with choline acetyltransferase (49) and has been implicated in cell injury recovery processes in neurons. Rat dorsal root ganglion (DRG) were incubated with various forms of beta-amyloid and a decrease in galanin immunoreactive neurons was identified (41). Cytokines and galanin have been suggested to function in a molecular cascade mediating injury-induced regeneration (29). Peripheral nerve damage upregulates cytokine IL-6 in DRG

neurons and these changes increase the levels of galanin in the DRG neurons. The increased levels of galanin in sensory neurons contributes to the initiation and maintenance of axonal regeneration in injured neurons (29).

GAP43, which was induced by the drugs, encodes a protein that localizes at the growth cone of neurite outgrowths. In astrocytoma tumors it is involved in attachment, spreading and motility (24). Cellular changes in GAP43 and galanin protein immunoreactivity have been studied in an axonal injury model (30). The axonal injury model revealed that neurons in the middle and caudal part of the ganglia survived the injury, and showed an increase in GAP43 and galanin immunoreactivity, indicating a sign of regeneration/neuronal plasticity (30). In AD patients GAP43 levels were decreased, suggesting in part that synaptic injury in the frontal cortex is an early event in AD (42).

Since galanin is a secreted neuropeptide, we hypothesize that it could act as autocrine survival factor. In fact, we found that exogenous galanin alone blocked the H_2O_2 induced cell death of the SH-SY5Y cells (Fig 3). Thus, galanin can act as a neuroprotective factor and should be considered for its therapeutic potential in treating neurodegeneration. Moreover, the galanin receptor could represent a therapeutic target for small drug discovery. These results have important ramifications for understanding and developing therapeutics for neuronal repair and preventing further oxidant-induced neural damage.

Collectively, our studies have identified a set of highly protective drugs that blocked oxidant-induced injury in SH-SY5Y cells, a small set of genes that were highly upregulated and downregulated by the protective compounds, and the specific involvement of the neuropeptide galanin in blocking oxidant injury to SH-SY5Y cells.

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Figure Legends

Figure 1:

Drug inhibition of low dose hydrogen peroxide induced oxidant injury. SH-SY5Y cells were incubated with 30 μM megestrol, 60 μM meclizine, 30 μM verapamil, 100 μM methazolamide, 10 μM sulindac, or 10 μM retinol for 24 hr. The drugs were removed and the cells were incubated with 100 μM hydrogen peroxide for 24 hr, and cell viability measured. Bars represent percent protection as compared to the controls in which no H_20_2 was added (± SDEV, n = 4-8).

Figure 2:

mRNAs differentially expressed in response to the drugs. Data expressed as gene expression ratios of $\log_2$(drug/control) across the drug treatments. The points on the graph represent the $\log_2$(drug/control) ratio across the drug treatments (n=2) (n=1 30 μM megestrol).

Figure 3:

Inhibition of oxidant-induced injury by the neuropeptide galanin. SH-SY5Y cells were incubated with galanin for 24 hr, followed by 100 μM hydrogen peroxide for 24 hr, and then cell viability was measured. Bars represent percent protection as compared to the controls in which no hydrogen peroxide was added. Data represent mean (± SDEV, n = 4-6).

Table 1

Cytoprotective drugs identified in the initial screen

Table 1

Class 1	**Class 2**	**Class 3**
Antibiotic	**Antihistamine**	**Ca Channel Blocker**
Erythromycin	Meclizine	Verapamil
Minocycline	Scopolamine	Nimodipine
Kanamycin		Nifedipine
Pramoxine		
Troleandomycin		
Sulfactamide		

Class 4	**Class 5**	**Class 6**
Steroid hormone	**Diuretic**	**NSAIDs**
Norethindrone	Methazolamide	Sulindac
Megestrol		
Dithranol		
Flunisolide		

Class 7	**Class 8**
Antiarrhythmics	**Miscellaneous**
Mexletine	Naltrexone (opioid antagonist)
Timol	Mercatoethanosufonic acid
Sotalol	Lithium (anti-despressant)
	Trazodone(anti-despressant)
	Bromocriptine (Parkinson’s disease)
	Retinol (topical vitamin)

Table 2

Cytoprotective drugs with high dose H_20_2

Table 2

Drug	Known Drug Action	Optimal dose	% Cytoprotection
Megestrol	Steroid hormone	30 μM	91
Meclizine	Anti-histamine	60 μM	98
Verapamil	Ca-channel blocker	60 μM	97
Methazolamide	Diuretic	100 μM	53
Sulindac	Anti-inflammatory	10 μM	70
Retinol	Vitamin	10 μM	77

Figure 1

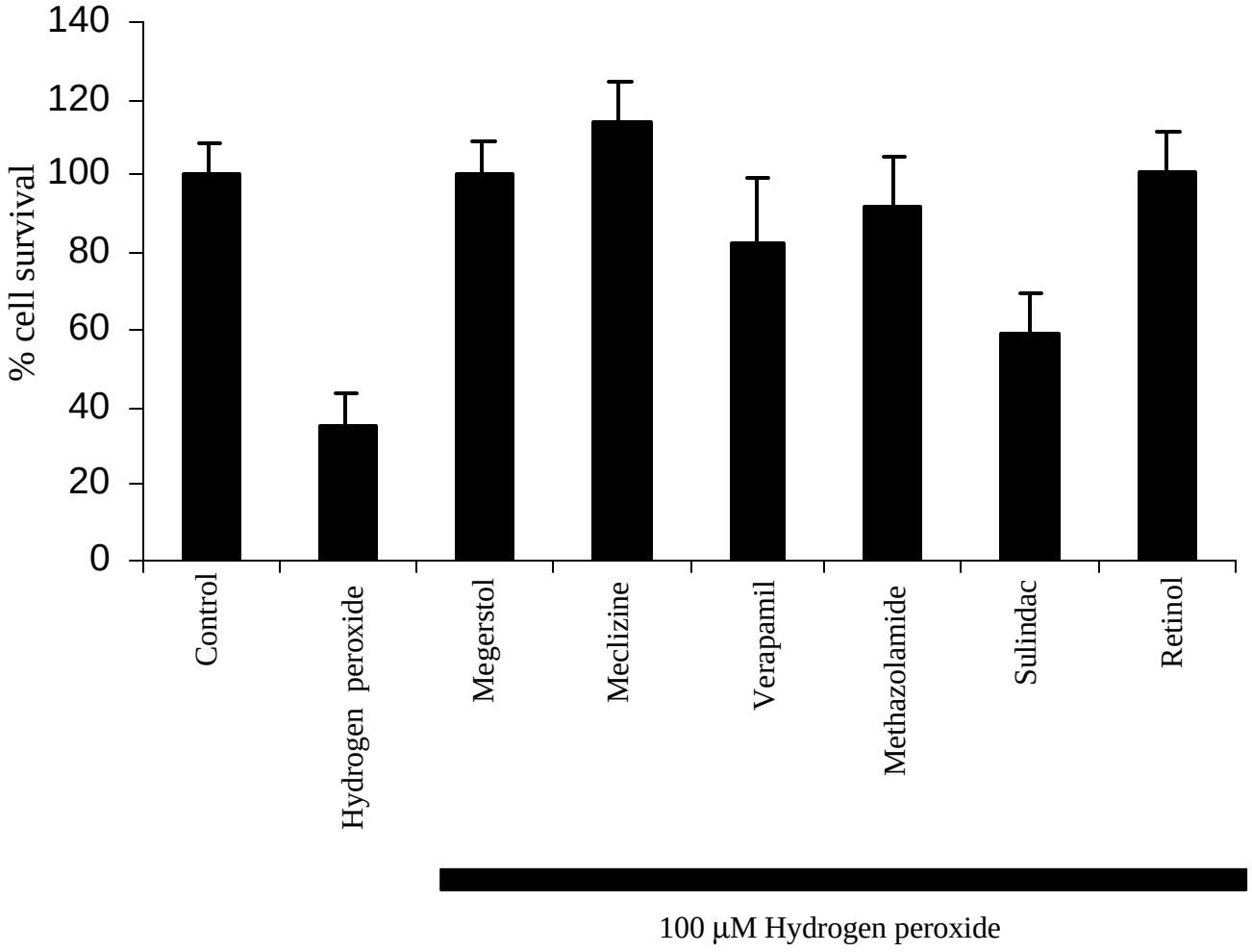

140
120
100
80
60
40
20
0
% cell survival
Control
Hydrogen peroxide
Megerstol
Meclizine
Verapamil
Methazolamide
Sulindac
Retinol
100 μM Hydrogen peroxide

Figure 2

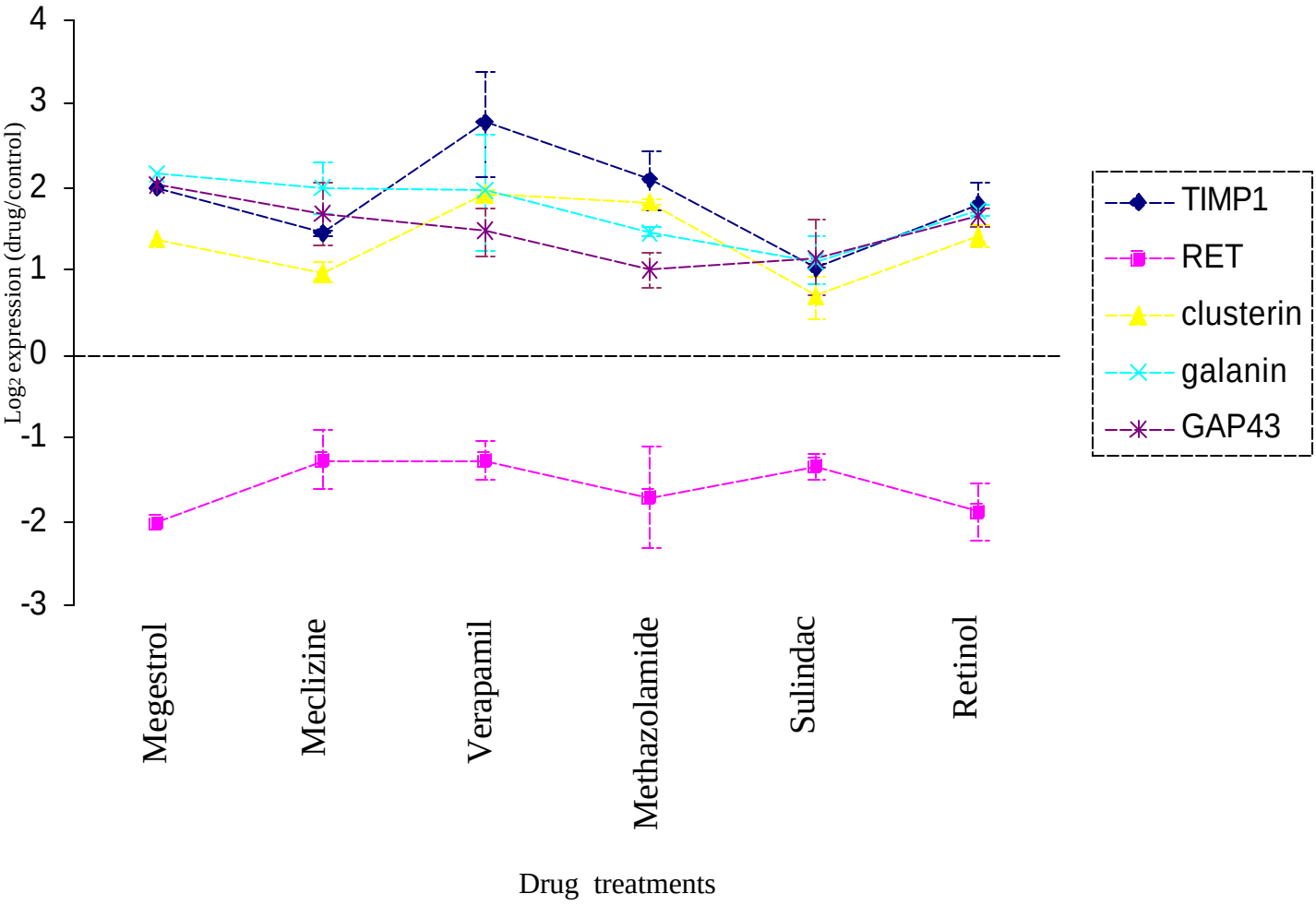
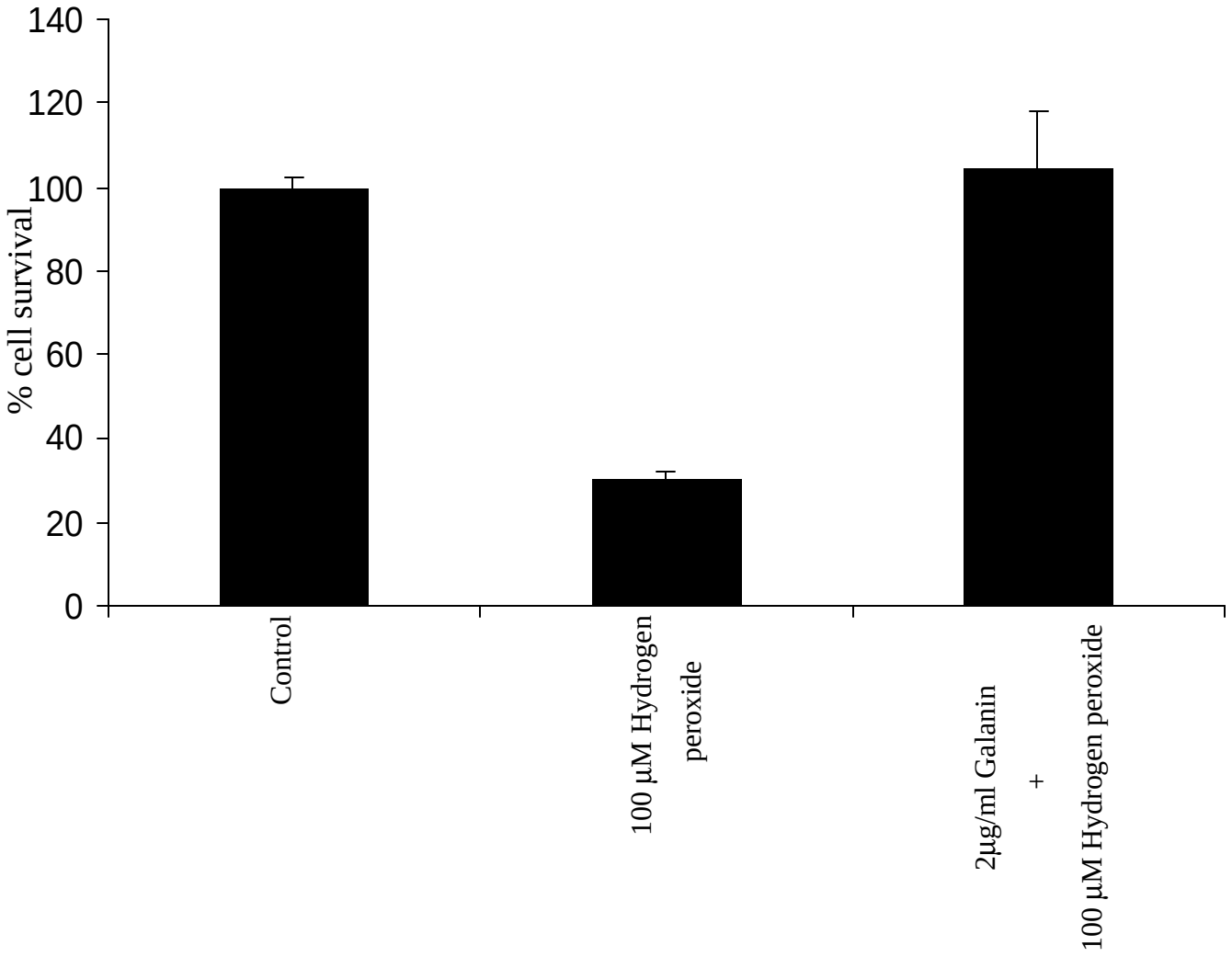

4
3
2
1
0
-1
-2
-3
Log2 expression (drug/control)
TIMP1
RET
clusterin
galanin
GAP43
Megestrol
Meclizine
Verapamil
Methazolamide
Sulindac
Retinol
Drug treatments

Figure 3


140
120
100
80
60
40
20
0
% cell survival
Control
100 µM Hydrogen peroxide
2µg/ml Galanin
+
100 µM Hydrogen peroxide