

Multiplex Gene Expression Analysis for High-Throughput Drug Discovery: Screening and Analysis of Compounds Affecting Genes Overexpressed in Cancer Cells¹

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Abstract

Drug discovery strategies are needed that can rapidly exploit multiple therapeutic targets associated with the complex gene expression changes that characterize a polygenic disease such as cancer. We report a new cell-based high-throughput technology for screening chemical libraries against several potential cancer target genes in parallel. Multiplex gene expression (MGE) analysis provides direct and quantitative measurement of multiple endogenous mRNAs using a multiplexed detection system coupled to reverse transcription-PCR. A multiplex assay for six genes overexpressed in cancer cells was used to screen 9000 chemicals and known drugs in the human prostate cancer cell line PC-3. Active compounds that modulated gene expression levels were identified, and IC₅₀ values were determined for compounds that bind DNA, cell surface receptors, and components of intracellular signaling pathways. A class of steroids related to the cardiac glycosides was identified that potently inhibited the plasma membrane Na⁺K⁺-ATPase resulting in the inhibition of four of the

prostate target genes including transcription factors Hoxb-13, hPSE/PDEF, hepatocyte nuclear factor-3 α , and the inhibitor of apoptosis, survivin. Representative compounds selectively induced apoptosis in PC-3 cells compared with the nonmetastatic cell line BPH-1. The multiplex assay distinguished potencies among structural variants, enabling structure-activity analysis suitable for chemical optimization studies. A second multiplex assay for five toxicological markers, Hsp70, Gadd153, Gadd45, O⁶-methylguanine-DNA methyltransferase, and cyclophilin, detected compounds that caused DNA damage and cellular stress and was a more sensitive and specific indicator of potential toxicity than measurement of cell viability. MGE analysis facilitates rapid drug screening and compound optimization, the simultaneous measurement of toxicological end points, and gene function analysis.

Introduction

Genomics-based strategies for identifying genes involved in disease processes are providing many new potential targets for therapeutic intervention (1, 2). However, the time and resources required to develop a protein or activity-based high-throughput assay for screening chemical compounds affecting a gene whose function has not been identified or fully characterized are a major rate-limiting step in drug discovery. Traditional drug discovery strategies typically use single end point screens and require extensive prior characterization of the gene product for assay development. Drug safety is usually evaluated only on optimized compounds.

One of several genomics-based strategies for discovering new drug target candidates for cancer therapy involves identifying genes that are differentially expressed in a tumor- and/or tissue-specific way (3, 4). The use of microarray technology to analyze the expression of human genes at the level of mRNA is having an important impact on cancer biology, pharmacology, and drug development (5). Potential target genes that are overexpressed in tumors can be further validated by demonstrating that a mRNA-specific knockdown (using antisense reagents) leads to a phenotypic effect as measured by a cell-based assay (e.g., inhibition of proliferation, induction of apoptosis). The functions of novel target genes that meet these criteria are usually unknown. Our challenge has been to develop a strategy that would avoid the considerable time and resources required to first identify the function of the target, develop biochemical assays for individual target gene proteins, and then run these assays separately. We demonstrate in this report the development

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and validation of a novel MGE⁹ technology for multitarget drug discovery. MGE assays permit high-throughput screening of chemical libraries and known drugs, characterization of compounds for potential toxicological effects, and quantitative measurement of drug dose-response that enable chemical optimization of lead compounds and establishment of SARs. A MGE assay for six genes overexpressed in prostate cancer cells identified a class of steroid drugs that are potent inhibitors of the plasma membrane Na⁺K⁺-ATPase (6), a potential target for prostate cancer therapy (7). The results also provided new information on the function of the prostate target genes by virtue of their response to drugs affecting known signaling pathways.

Materials and Methods

Cell Culture and Compound Treatment. PC-3, a human prostate adenocarcinoma cell line, was cultured at 37°C and 5% CO₂ using Kaighn's Nutrient Mixture F-12 (Irvine Scientific, Santa Ana, CA) containing 7% fetal bovine serum and 1 mM L-glutamine. Lidded 96-well microtiter culture plates were seeded at 5 × 10⁴ cells/well in 200 μl of media and incubated for 3 h (37°C, 5% CO₂) before compound addition. A set of 80 known drugs ("Killer" Plate 1; MicroSource Discovery Systems, Inc., Gaylordville, CT) and the Act-D positive control were solubilized in 100% DMSO and diluted to 8× working solutions with growth media before cell plate addition. Compounds from a chemical library (in pooled format) and individual compounds were analyzed at a final concentration of 2.5 μM in 0.25% DMSO. This DMSO concentration had no effect on cell growth or target gene expression. For dose-response analysis, compounds were plated in triplicate and analyzed using eight concentrations (10 μM to 3.16 nM in 0.25% DMSO), serially diluted. After cell attachment was verified by phase-contrast microscopy, a 25-μl aliquot of media was removed, and an equivalent volume of compound working solution (8×) was introduced with mild trituration and incubated for 24 h.

Cell Lysis and RNA Sample Preparation. Lysis buffer was prepared by adding external mRNA controls to a final concentration of 500 fM and 145 mM β-mercaptoethanol in RLT lysis buffer (Qiagen, Valencia, CA). Two external mRNA controls were used: 7.5-kb poly(A)-tailed RNA (Life Technologies, Inc., Gaithersburg, MD) and 1.2-kb kanamycin positive control [Promega, Madison, WI (both DNase-treated)]. After the 24-h incubation period, cell media were aspirated from all wells, and lysis buffer (100 μl) was added to each well containing cells. Plates were then mixed for 15 s and sealed before storage at -20°C. Cell lysates were thawed, and total RNA was purified in automated 96-well format using the Qiagen RNeasy 96 kit according to the manufacturer's instructions. RNA concentrations were determined fluorometrically using RiboGreen reagent (Molecular Probes, Eugene, OR), automatically adjusted in concentration, and

aliquoted in 30-ng amounts into 96-well plates for assay. Total RNA yields ranged from 0.45 to 1.8 μg/well, depending on compound toxicity.

Cell Viability and Compound Toxicity. Evaluation of compound cytotoxicity was determined by measuring cell viability using the WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay (8). Assay conditions were the same as those described above. At the end of the incubation period, 5% (v/v) WST-1 reagent (Roche Molecular Biochemicals, Indianapolis, IN) was added to each well. Cells were then incubated at 37°C, 5% CO₂ for an additional hour. Plates were mixed for 1 min, and absorbance values were read at 440 nm. Test compound concentrations were considered cytotoxic if metabolic response (percentage viability) was less than 80% that of vehicle control wells.

MGE Assays. Two MGE assays were developed, one to quantify the levels of six prostate cancer target genes, and a second to quantify expression levels of five genes indicative of DNA damage and cellular stress. In addition to the target genes, each multiplex was designed to measure levels of two internal control genes, β-actin and GAPDH, and two spiked external RNA controls. Two of the controls were endogenous cellular mRNAs that exhibit relatively constant expression levels (β-actin and cyclophilin), used for normalization of signals from other genes. Two additional control RNA targets were added exogenously in the cell lysis buffer to provide a means to monitor recovery and stability of RNA from cell lysates (kanamycin mRNA and the 7.5-kb RNA).

The multiplex assay protocol utilizes solution-phase quantitative multiplex RT-PCR amplification coupled with multiplexed fluorescence readout. Primer pairs for target genes and reference sequences were designed using Primer-3 software (Whitehead Institute for Biomedical Research) and are shown in Table 1. Each primer consisted of two main regions, a 5' region complementary to a common universal sequence and a 3' region designed to hybridize specifically with the gene of interest. Forward primers contained the SP6 universal sequence and were 5'-labeled with 6-carboxyfluorescein (6-FAM; Glen Research, Sterling, VA). Reverse primers contained the T7 universal sequence. The universal sequences allowed uniform amplification of all of the gene-specific targets using the same universal primers because early cycles of amplification result in the addition of the universal sequences to the flanking regions of the amplification products, creating a template that is amplified by the universal primers alone. Primers were designed such that amplification products for each target gene were of different sizes, permitting subsequent electrophoretic separation. The sizes of the predicted PCR amplification products of the 17 target sequences ranged from approximately 100 to 330 bp, with the smallest size difference being 5 bp. Primers were synthesized by Operon Technologies Inc. (Alameda, CA) using conventional phosphoramidite chemistry.

Assays were run in 96-well format, with each plate containing RNA from a series of cells dosed with individual drugs or pooled compounds and controls (Fig. 2a). Reverse transcription to generate first-strand cDNA was carried out using 30 ng of total RNA, 0.02 μM reverse target-specific primers,

⁹ The abbreviations used are: MGE, multiplex gene expression; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Act-D, actinomycin D; CV, coefficient of variation; MGMT, O⁶-methylguanine-DNA methyltransferase; TSA, trichostatin A; RA, retinoic acid; SAR, structure-activity relationship; HNF-3α, hepatocyte nuclear factor-3α.

Table 1 Primer sequences

Accession no.	Primer	Size (bp)	Forward primer sequence	Reverse primer sequence
X00351	β -Actin	117	AGGTGACACTATAGAATAACCG ATAAGGCCAACCGCGAGAAGA TGA	GTACGACTCACTATAGGGATGGA TAGCAACGTACATGGCTG
U02426 fragment	7.5 kb	127	AGGTGACACTATAGAATAACTA TGCCGGTATCAGCACC	GTACGACTCACTATAGGGAGATG GCAGCGTATTTACAC
Ina D	Ina D	147	AGGTGACACTATAGAATAGTGA CACGTGCGAGAATGAG	GTACGACTCACTATAGGGATTGA CCCTTCAGTTGCTTGA
hSPE	hSPE	157	AGGTGACACTATAGAATAGCTT CATTAGGTGGCTCAACA	GTACGACTCACTATAGGGAGGCT CAGCTTGTCTGATGTTT
Survivin	Survivin	200	AGGTGACACTATAGAATAGTCA GCCCAACCTTCACATC	GTACGACTCACTATAGGGACCAC CCTGCAGCTCTATGAC
HNF-3 α	HNF-3 α	215	AGGTGACACTATAGAATAACTT CAAGGCATACGAACAG	GTACGACTCACTATAGGGAGGGA GCTAGGAAGTGTTTAG
M33197	GAPDH	237	AGGTGACACTATAGAATAAAG GTGAAGGTCCGAGTCAA	GTACGACTCACTATAGGGAAATGA CAAGCTTCCCCTTCTC
Blx-33	Blx-33	266	AGGTGACACTATAGAATAGCTC ATCTGCCAACAAATC	GTACGACTCACTATAGGGACTAG CGGAAGCAAATTACAC
Hoxb-13	Hoxb-13	283	AGGTGACACTATAGAATAGCG ACATGACTCCCTGTT	GTACGACTCACTATAGGGAAACT TGTTAGCCGCATACTC
J01839 (V00359)	KanR	322	AGGTGACACTATAGAATAATCA TCAGCATTGCATTGATTCTGTTTG	TACGACTCACTATAGGGAAATCC GACTCGTCCAACATC
X00351	β -Actin	110	AGGTGACACTATAGAATA AAGGCCAACCGCGAGAAG ATGA	GTACGACTCACTATAGGGA TGGATAGCAACGTACATGGCT G
S40706	Gadd153	140	AGGTGACACTATAGAATA ACAGAGTGGTCATTCCCAG	GTACGACTCACTATAGGGA GCCGTTTATTCTTTCAGCT
M60974	Gadd45	163	AGGTGACACTATAGAATA AACGGTGATGGCATCTGAAT	GTACGACTCACTATAGGGA CCCTTGGCATCAGTTTCTGT
M29971	MGMT	172	AGGTGACACTATAGAATA ACCGTTTGGACTTGGTACT	GTACGACTCACTATAGGGA CCTTGCCAGGAGCTTTATT
U94788	p53	186	AGGTGACACTATAGAATA GCATGGGCGGCATGAACC	GTACGACTCACTATAGGGA TCTTGCGGAGATTCTCTTC
Y00052	Cyclo	268	AGGTGACACTATAGAATA GCGTCTCCTTTGAGCTGTTT	GTACGACTCACTATAGGGA CCAGGACCGTATGCTTTAG
L12723	Hsp70	306	AGGTGACACTATAGAATA AAGAGCAACAGCAGCAGA CA	GTACGACTCACTATAGGGA CCACTGCGTTCTTAGCATCA
	SP6 universal T7 universal		AGGTGACACTATAGAATA GTACGACTCACTATAGGGA	

1 mM deoxynucleotide triphosphates, RNasin RNase inhibitor (2.5 units, Promega), and Moloney murine leukemia virus reverse transcriptase (10 units; Promega) in 20 mM Tris, 16.7 mM magnesium chloride (pH 8.3). Samples were incubated at 42°C for 30 min, followed by 95°C for 5 min to inactivate the enzyme. The cDNA products from the reverse transcriptase reaction were used directly as templates in the subsequent PCR amplification. PCR amplifications were carried out with a mixture of forward gene-specific primers (0.01 μ M), 5'-FAM-labeled forward universal primer (0.5 μ M), reverse universal primer (0.5 μ M), deoxynucleotide triphosphates (0.375 mM; Promega), and AmpliTaq Gold polymerase (1 unit, Perkin-Elmer, Foster City, CA) in the buffer supplied with the enzyme. Thermal cycling was carried out under conditions to maintain amplification in the exponential range of the reaction on a Perkin-Elmer GeneAmp 9700 over 35 cycles using the following conditions: 94°C for 30 s; 55°C for 30 s; and 68°C for 1 min.

Multiplex PCR products were resolved by gel electrophoresis on a standard of 5% polyacrylamide sequencing gel containing 6 M urea and 890 mM Tris-borate and 2 mM EDTA

with an ABI PRISM 377 DNA Sequencer (Perkin-Elmer/Applied Biosystems). Amplification products were diluted and mixed with a solution of GeneScan 500 ROX-labeled size standards (Perkin-Elmer/Applied Biosystems) in formamide (1:5) and loaded on the gel. The components of the multiplex reaction mixture were electrophoretically separated by size by running for 1.5 h at 2000 V, 60 mA current, 20 W power, gel temperature of 51°C, and laser power of 40 mW (ABI 377). Fluorescence data were collected by laser scanning across the gel in real time. GeneScan software was used to quantitate fluorescent signals from the amplification products, and Genotyper software (both from Perkin-Elmer/Applied Biosystems) was used for assigning data from the amplification products to defined size ranges relative to the ROX standards. Quantitative levels of amplification products for the genes in each sample, including target and external spike control genes, were normalized to the internal controls and expressed as ratios to the housekeeping genes β -actin and GAPDH.

Multiplexed amplifications were validated to ensure that each primer pair (chosen from several possibilities) was spe-

cific for a particular target sequence and that there were no interactions among the target sequences/primer sets in the multiplex. This was accomplished by conducting dropout experiments, in which the multiplex amplification was run in the absence of all possible primer pairs. Additionally, the amplification reaction was validated by comparing the results of primers alone and in different multiplex environments, ensuring identical PCR products in each case.

Deconvolution of Pooled Compounds and Dose-Response Analysis. Compounds were screened using a chemical library (Berlex Biosciences, Richmond, CA) in which the compounds were formatted in a pool matrix (9) combining 10 individual compounds in each sample well. A compound appeared twice within a pool set, each time with a completely different set of accompanying compounds. Individual compound locations were mapped and correlated with their respective well activities in a database. These data were then deconvoluted (9) using a software program to identify active compounds and provide activity-ranking tables that were filtered and sorted.

Compound activity was defined as percentage inhibition = $(1 - (\text{sample well ratio}/\text{vehicle control well mean ratio})) \times 100$, where ratio = gene target RNA relative fluorescence units/ β -actin RNA relative fluorescence units. Compounds that yielded percentage inhibition greater than 50% and a δ value (difference between duplicate activities) less than either 50% or 10% were selected for individual compound activity confirmation. Confirmed actives were then subjected to dose-response analysis, and data were analyzed using conventional methodologies. IC_{50} values were determined using a four-parameter logistic fit that allows for a variable slope.

Apoptosis Assay. Selected compounds identified by high-throughput screening were evaluated for their ability to selectively induce apoptosis in PC-3 cells as measured by internucleosomal DNA fragmentation in suspension-cultured PC-3 prostate cancer cells using a modified double filter binding assay described previously (10). Cellular DNA was analyzed by harvesting compound-treated, [^3H]thymidine-labeled PC-3 cells onto two filters, a glass fiber filter and a DEAE filter. The glass fiber filter trapped intact chromatin and high molecular weight DNA, whereas the DEAE filter trapped the low molecular weight DNA fragments. Apoptosis, as measured by DNA fragmentation, was evaluated by quantitating the ratio of radioactivity retained on each of the filters.

PC-3 prostate adenocarcinoma cells and BPH-1 benign prostatic hyperplasia adherent cells were labeled as follows. PC-3 cells required serum starvation for 24 h prior to addition of [^3H]thymidine. At labeling time, complete medium containing [^3H]thymidine (~ 80 Ci/mmol; 25 $\mu\text{Ci}/1 \times 10^7$ cells) was added to the cells (PC-3 and BPH-1) and the cells were then incubated for 4 to 24 h (incubation time dependent on cell type). [^3H]thymidine labeled cells were trypsinized and plated in 96-well plates (1×10^4 or 5×10^4 cells/well depending on cell type). The cells were allowed to recover overnight. Cisplatin control (30–100 μM range) or test compounds were subsequently added to the cells and incubated for 48 to 72 h (incubation time was dependent on cell type). The final assay volume was 150 μl per well. Following incubation, cells were

lysed with equal volumes of lysis buffer (0.2% Tween-20, 20 mM EDTA, 20 mM Tris-HCl, pH 8.0) for 0.5 h and then harvested through a Packard plate harvester containing two filter mats; the lower being a glass (neutral) fiber filter mat and the upper a DEAE (positively charged) filter. The high molecular weight DNA and chromatin were captured by the glass fiber filter while the low molecular weight DNA fragments were trapped by the DEAE filter. The filters were then separated and dried. DNA fragmentation was quantified by counting the radioactivity retained by each of the filters and taking the ratio between the two filters.

Results

Assay Design, Reproducibility, and Sensitivity. Six prostate target genes comprising the multiplex assay consisted of three transcription factors and three non-transcription factors: (a) the homeodomain transcription factor, Hoxb-13 (11); (b) an Ets transcription factor family member, hPSE/PDEF (12, 13); (c) the HNF-3 α transcription factor (14); (d) survivin, an inhibitor of apoptosis family member (15, 16); (e) Ina D, a gene coding for a PDZ domain-containing protein (17); and (f) a novel gene of unknown function, designated Blx-33, involved in apoptosis induction. Each gene was determined to be overexpressed in prostate or other tumor tissue relative to normal or nonprostate tissues. Additionally, inhibition of gene expression by antisense oligonucleotides for the individual target genes was demonstrated to result in the inhibition of cell proliferation in PC-3 cells (data not shown). The general assay protocol (Fig. 1a) consisted of six fully automated steps in 96-well microtiter plate format: (a) cell plating and drug treatment; (b) cell lysis and RNA isolation; (c) RNA quantitation, normalization, and sample archiving; (d) quantitative RT-PCR multiplex reaction; (e) fluorescence detection; and (f) data deconvolution.

Assay reproducibility (Fig. 1b) was determined by measuring gene expression levels in RNA samples obtained from three replicate wells of PC-3 cells dosed with vehicle control in each of twenty 96-well assay plates. Intraplate CVs decreased slightly with increasing gene expression level, ranging from 14% for low expression level genes such as Ina D (0.03 relative to β -actin) to 9.5% for high expression level genes such as hPSE (0.35 relative to β -actin). Overall process CVs ranged from 34% for low expression level genes to 18% for high expression level genes.

RT-PCR methodology is the most sensitive method currently available for transcript profiling (18). To assess the sensitivity and dynamic range of RNA detection, a 7.5-kb RNA was spiked into 30 ng of total RNA from cultured PC-3 cells in the range of 0.004 to 125 attomoles. Each spiked sample was assayed in triplicate in the multiplex assay, and the quantities of specific PCR products, relative to β -actin, were determined. The dose-response was approximately linear over at least 2 orders of magnitude (Fig. 1c). The expression levels of the nine other genes were unaffected at all doses of the spiked RNA (data not shown). The minimum detectable level of spiked 7.5-kb RNA that could be reproducibly distinguished from 0 was 63 zeptomoles, or 4×10^4 molecules. These data indicated that the assay could detect approximately one 7.5-kb transcript copy per cell in 50,000

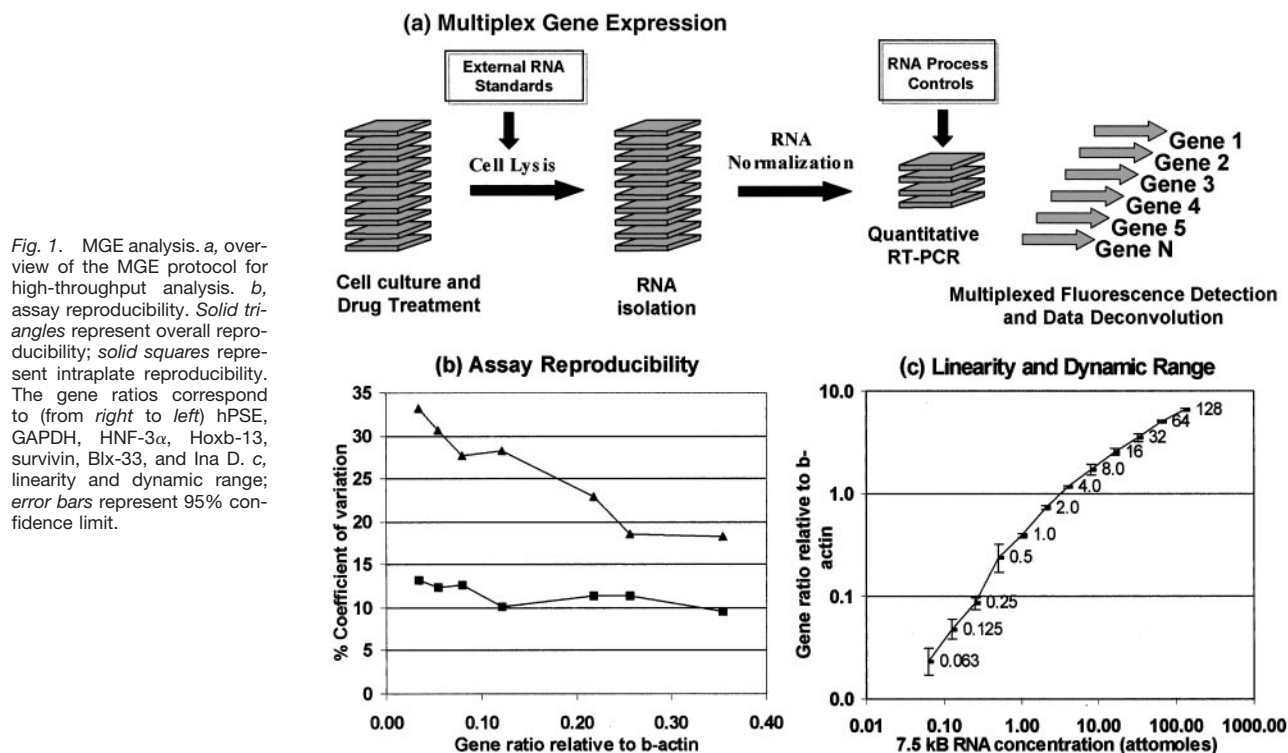


Fig. 1. MGE analysis. *a*, overview of the MGE protocol for high-throughput analysis. *b*, assay reproducibility. *Solid triangles* represent overall reproducibility; *solid squares* represent intraplate reproducibility. The gene ratios correspond to (from right to left) hPSE, GAPDH, HNF-3 α , Hoxb-13, survivin, Blx-33, and Ina D. *c*, linearity and dynamic range; *error bars* represent 95% confidence limit.

cells (per well). Due to variations in PCR efficiency, however, the sensitivity of the assay can vary for different genes. In the current assay format, it is generally on the order of a few copies per cell using 30 ng of input RNA.

Two types of experiments were performed to validate the changes in mRNA levels determined by MGE assays. Using specific antisense reagents to Hoxb-13, hPSE, and Blx-33, we demonstrated that independent analysis using TaqMan technology resulted in comparable changes in mRNA levels (>50% decrease). In addition, we measured the percentage change in gene expression (mRNA levels) as a function of time of treatment with 2 μ M Act-D using the MGE assay. Northern hybridization analysis demonstrated similar results (data are presented in Supplementary Material I).¹

Screening Known Drugs. The ability of the prostate target multiplex assay to identify compounds that inhibit or stimulate specific gene expression was evaluated by screening a set of 80 known drugs representing many different mechanistic classes (see Supplementary Material II)¹ and by showing that IC₅₀ values could be reproducibly measured for selected active compounds. Twenty-five of the 80 compounds were active, 18 of which are shown in Table 2. The results define several distinct expression profiles associated with different drugs as represented by genes whose expression is inhibited (Hoxb-13, hPSE, HNF-3 α , and survivin) and other genes that are either inhibited or stimulated (Blx-33 and Ina D). Some inhibitors of RNA and protein synthesis (Act-D, cycloheximide, emetine, and puromycin) have fairly broad effects on the prostate target genes, whereas others (acri-flavinum, aminopterin, chlorotetracycline, and quinidine) are more gene selective under the assay conditions. Drugs that

affect tubulin (colchicine, cytochalasin D, podophyllotoxin, and vinblastine sulfate) predominantly inhibited the transcription factors. Drugs affecting mitochondrial respiration (antimycin A, gramacidin, and rotenone), protein and nucleic acid synthesis (aminopterin, emetine, and puromycin), and a DNA alkylating (cross-linking) agent (mitomycin C) all caused an up-regulation of Blx-33, suggesting a role for this unknown gene in DNA damage and stress response. Two drugs, digitoxin and ouabain, representative of cardiac glycosides that are specific inhibitors of the Na⁺K⁺-ATPase (sodium pump), were found to be the most potent inhibitors of the three transcription factors (Hoxb-13, hPSE, and HNF-3 α).

IC₅₀ Analysis, Drug Kinetics, and mRNA Stability. Based on the data from the screen with the known drug compounds, Act-D, a DNA intercalator that inhibits RNA synthesis and the expression of the prostate target genes, was chosen to serve as the positive control for high-throughput screening of the chemical library. It was also used to estimate transcript stabilities to choose an appropriate drug treatment time in which to detect compounds that inhibit transcription. To evaluate the potency of Act-D on each of the target genes, the prostate target multiplex assay was used to measure Act-D dose-response kinetics (Fig. 2a). IC₅₀ values were measured five times independently and demonstrated a high degree of reproducibility (e.g., 13.8 \pm 0.84 nM, 26 \pm 2.9 nM, and <5 nM for hPSE, HNF-3 α , and Hoxb-13, respectively). The *top panel* of Fig. 2a shows that Act-D inhibition of RNA synthesis increases the ratio of 7.5-kb control RNA to β -actin by about 2.5-fold at all concentrations. Even at 10 nM Act-D, there is an approximately 50%

Table 2 Expression profiles of active compounds

Compounds were assayed in triplicate at 2 μM for 24, 36, and 48 h; % Inhibition is the mean value of the gene expression change at 36 h relative to vehicle control, except ^a is for 48 hrs. +, increase in gene expression; -, no detectable change; <, less than 40% inhibition.

Drug	% Inhibition (gene expression)					
	Hoxb-13	hPSE	HNF-3 α	Blx 33	Ina D	Survivin
Acriflavium	59	<	43	50	<	<
Act-D	67	47	76	59	<	<
Aminopterin	-	-	-	-	40	47
Antimycin A	-	-	-	+142 ^a	-	55
Chlorotetracycline	66	-	<	58	+211	-
Colchicine	57	-	44	-	+106	-
Cycloheximide	70	56	<	+73	66	77
Cytochalasin D	50	50	40	<	<	42
Digitoxin	100	62	66	57	+76	-
Emetine	69	59	-	+314	+55	-
Gramacidin	64	-	-	+71	+110	-
Mitomycin C	-	-	-	+62	-	-
Ouabain	100	87	76	50	52	50
Podophyllotoxin	63	40	60	-	63	-
Puromycin	50	77	+107 ^a	+68 ^a	-	57
Quinacrine	52	40	-	54	51	-
Rotenone	46	-	-	+87 ^a	-	45
Vinblastine sulfate	83	62	70	55	68	<

^a Mean value of the gene expression change at 48 h.

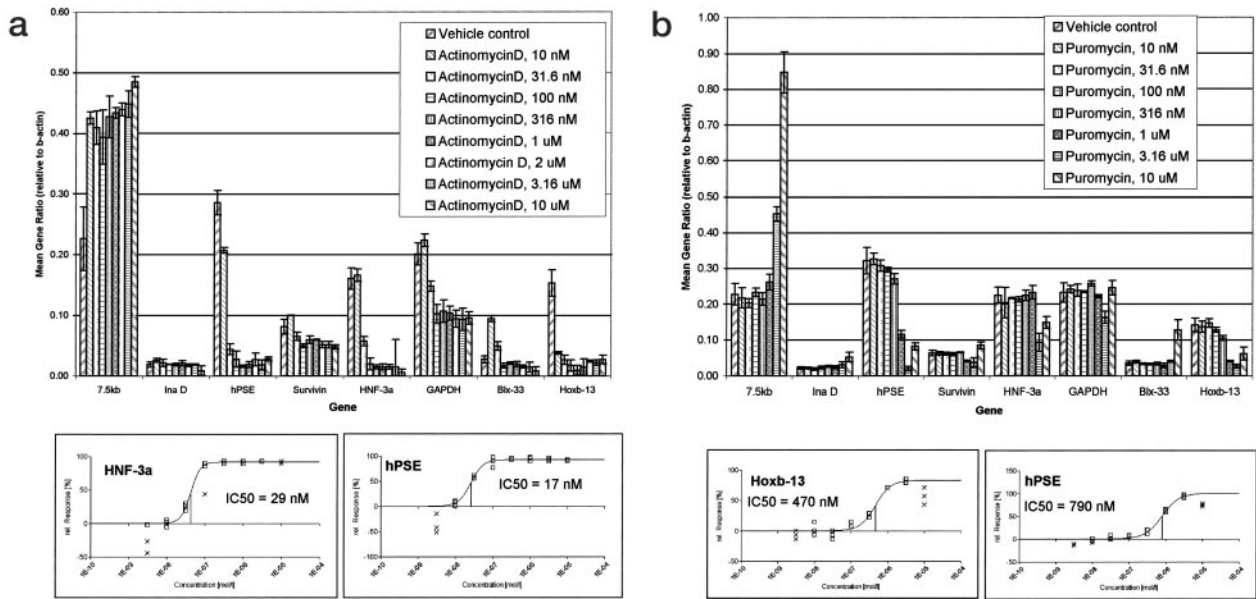


Fig. 2. a, Act-D IC₅₀ analysis. Top panel, gene expression ratios (relative to β -actin) calculated for the indicated target genes in PC-3 cells at the indicated drug concentrations after 24 h of treatment. Bottom panels, IC₅₀ curves for Act-D inhibition of hPSE and HNF-3 α . b, puromycin IC₅₀ analysis. Top panel, gene expression ratios (relative to β -actin) calculated for the indicated target genes in PC-3 cells at the indicated drug concentrations after 24 h of treatment. Bottom panels, IC₅₀ curves for Act-D inhibition of Hoxb-13 and hPSE.

inhibition of the β -actin mRNA level that correlates with a 42% reduction in total RNA yield (data not shown). Because the β -actin level remains constant throughout the dose range, the IC₅₀ calculation is not affected. In contrast to Act-D, puromycin does not affect the ratio of 7.5-kb control RNA to β -actin until the highest drug concentrations (3.16 and 10 μM) are reached. These high drug concentrations are outside the dose range necessary to calculate IC₅₀ values of

470 and 790 nM for Hoxb-13 and hPSE, respectively (Fig. 2b). Fig. 3 shows the kinetic effects of Act-D treatment, over time, on target gene expression over a broad dose range of drug. In general, the transcription factor genes, Hoxb-13, hPSE, and HNF-3 α , appear to have mRNAs that decay >90% within 24 h, whereas the non-transcription factor genes, Blx-33, survivin, and Ina D (data not shown), have more stable mRNAs. The transcription factor genes show sus-

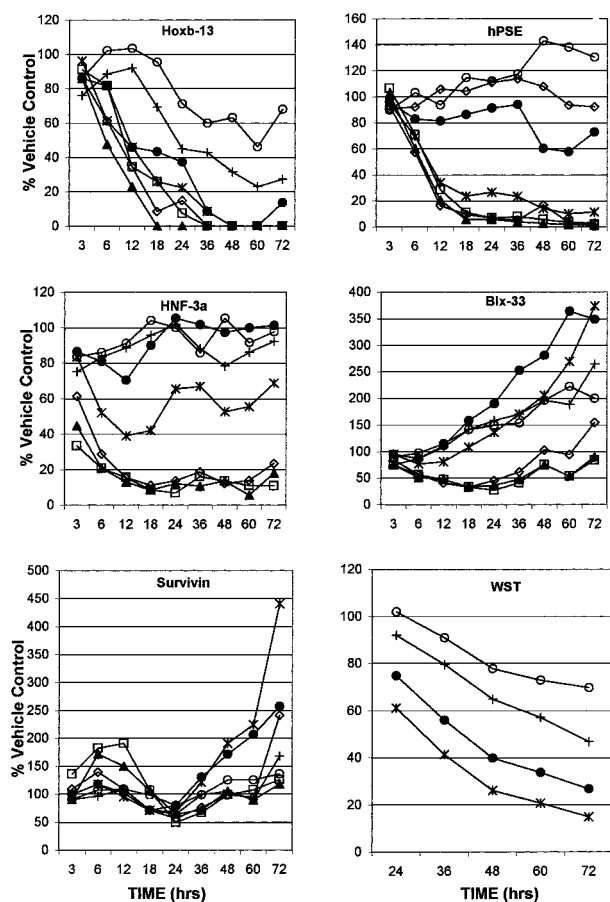


Fig. 3. Act-D inhibition kinetics of prostate target gene expression. Change in gene expression as a percentage of vehicle control (or WST-1 assay, *bottom right panel*) as a function of Act-D treatment time in PC-3 cells at the following drug concentrations: 1 nM, ○; 3.16 nM, +; 10 nM, ●; 31.6 nM, *; 100 nM, -; 316 nM, ▲; and 1 μ M, □.

tained but distinguishable inhibition effects at Act-D concentrations between 316 nM and 1 μ M, whereas survivin and Blx-33 have more complex kinetic patterns of both stimulation and inhibition. Act-D exhibits a pronounced dose-dependent toxicity between 1 and 31.6 nM (Fig. 3, *bottom right panel*) as measured by the WST-1 assay, a mitochondrial reductase assay used to monitor cell viability.

Pooled Compound Screening. From the analysis of known drugs (Table 2), we discovered that two cardiac glycosides, ouabain and digitoxin, were particularly potent inhibitors of Hoxb-13 and hPSE (IC_{50} values determined by the multiplex assay were in the range of 18–60 nM, as described below). To evaluate the ability of the assay to screen pooled compounds, a common format for large chemical libraries (9), we tested a set of twenty 96-well plates containing approximately 9000 compounds in pools of 10 compounds. Identification of the active compounds was determined by the deconvolution method (9) described in “Materials and Methods.” A single pool 10-compound collection, known to contain other cardiac glycosides, was chosen from the available chemical library. Cells were treated with the compound

Table 3 Active compounds in pool-10 format

Number of active compounds exhibiting greater than 50% inhibition (I), or greater than 70% I for Hoxb-13, in pool-10 format. δ numbers represent the difference in inhibition values between the two independent assays of a compound in pool-10 format. Confirm is the number of active compounds from the pool-10 format that are active in single compound format. Stability (mRNA) is the percentage of transcript remaining after 2 μ M treatment with Act-D for 24 h.

Target	Stability	Active compounds		
		$\delta < 50$	$\delta < 10$	Confirm
Hoxb-13	<5	58	92	30
hPSE	<5	51	87	25
HNF-3 α	<5	41	71	23
Blx-33	>50	4	0	1
Ina D	>50	3	0	1
Survivin	>90	7	0	2
GAPDH	>70	0	0	0

Table 4 Structure-activity analysis

Structure	Inhibition of gene expression IC_{50} (nM)				Apoptosis IC_{50} (μ M)	
	Hoxb-13	hPSE	HNF-3 α	Survivin	PC-3	BPH-1
1	21	38	31	20	0.70	>100
2	88	200	98	70	nd ^a	nd
3	270	560	380	230	nd	nd
4	1300	>5000	>5000	660	>30	>100
5	>5000	>5000	>5000	>5000	nd	nd
6	29	61	52	30	0.52	>100
7	66	120	84	59	0.63	>100
8	92	170	110	51	2.7	>100
9	100	300	310	63	5.3	>100
10	1800	4000	>5000	1500	>30	>100
C-1	nd	nd	nd	nd	1.8	4.5
C-2	nd	nd	nd	nd	1.5	0.4
Cisplatin	nd	nd	nd	nd	3.5	11

^a nd, not determined; compound 6 = ouabain.

library at a concentration of 2.5 μ M for 24 h, based on the results from the control screen and the Act-D experiments. Table 3 shows the number of active compounds at two different δ values (the difference in percentage inhibition values of a compound in two different wells) and the numbers of compounds that were confirmed by single compound, single dose analysis. We observed a correlation between the target gene’s mRNA stability and the “hit rate,” suggesting at least two mechanistic classes of compounds, those inhibiting transcription (major class with relatively short mRNA half-lives) and those affecting mRNA stability (minor class with relatively long mRNA half-lives).

Cardiac Glycosides and Structure-Activity Analysis. Results from screening the pool-10 plates identified an additional 34 confirmed hits with potencies that varied over an approximately 1000-fold range. Structure-activity data are presented in Table 4 for some representative compounds, including ouabain (compound 6; Fig. 4). Four of the prostate target genes, Hoxb-13, hPSE, HNF-3 α , and survivin, showed significant inhibition after 24 h of drug treatment, whereas Blx-33 was inhibited at later times (data not shown). Compounds (Fig. 4) were also evaluated for their ability to selectively induce apoptosis in PC-3 cells. Table 4 (right-hand

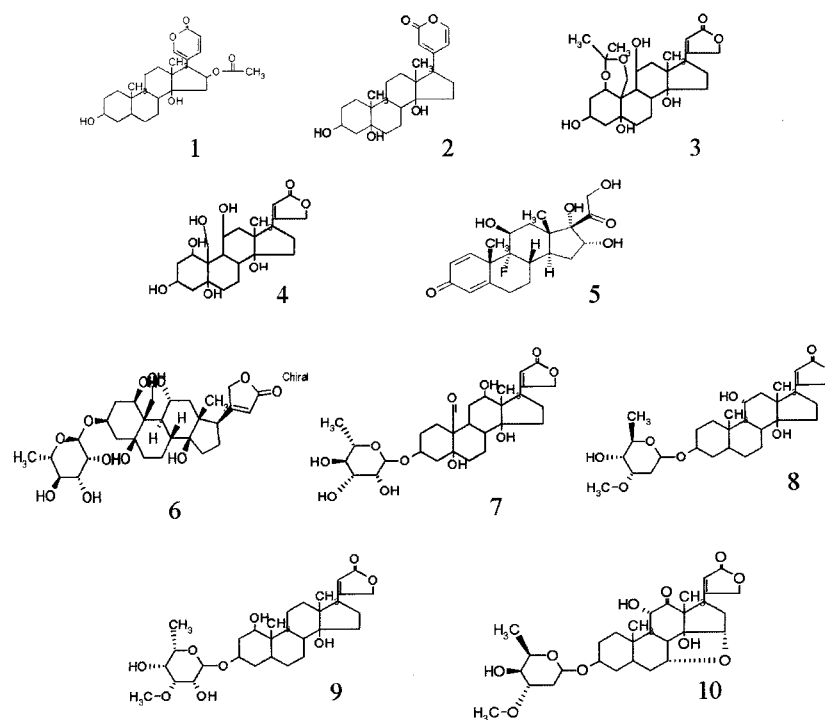


Fig. 4. Structure of compounds listed in Table 4.

columns) demonstrates that active steroids preferentially induce apoptosis in PC-3 cells compared with BPH-1 cells, and their ability to induce apoptosis correlates with their activity in inhibiting expression of Hoxb-13, hPSE, HNF-3 α , and survivin. Two nonsteroid control compounds, C-1 (a nucleoside analogue), C-2 (a phenyl-pyrrole analogue), and cisplatin, by comparison, induced apoptosis in both PC-3 and BPH-1 cell lines.

Cardiac glycosides consist of a core steroid structure with cis A,B and C,D ring junctions and typically a sugar moiety attached to C3 of the steroid ring. Cardiac glycosides have a distinctive and well-studied SAR (19), with a β -hydroxyl group on C14 and a β -unsaturated lactone on C17, both important for activity. The sugar on C3 is not required for activity; highly active compounds were found both with (compounds 1–5) and without (compounds 6–10) a sugar at C3, the latter represented by compounds with a 3- β -hydroxyl group. Both 5- and 6-membered unsaturated lactones were active. No compounds without this functionality were active, including compounds with a variety of linear 17- β substituents (data not shown). The SAR represented by the data of Table 4 is consistent with inhibition of Na⁺K⁺-ATPase as the mechanism by which these compounds inhibit expression of Hoxb-13, hPSE, HNF-3 α , and survivin and induce apoptosis.

Steroid structures 1 and 2 are bufadienolides, or bufalins (19), distinguished from the others by possession of a 6-membered lactone moiety at the 17 position, with potencies similar to the glycosides digitoxin and ouabain (IC₅₀ values for Hoxb-13 of 18 and 29 nM, respectively). Bufalins have been demonstrated to be potent inducers of apoptosis in human leukemic cells but not normal leukocytes (20). The

results of Table 4 also demonstrate that the prostate target multiplex assay can distinguish potencies among structural variants in a single chemical class. We have demonstrated this with other chemical classes as well (data not shown). The changes in potency with structural variation are generally similar for Hoxb-13, hPSE, HNF-3 α , and survivin, suggesting that these genes may be linked by a common pathway influenced by Na⁺K⁺-ATPase.

Toxicology Analysis. The set of 80 known drugs (Supplementary Material II)¹ was analyzed with a second multiplex assay comprising hsp70, gadd45, gadd153, MGMT, cyclophilin, and p53 (not expressed in PC-3 cells), genes that are well known to respond to DNA damage and cellular stress. (Total RNA yields were also measured, and the complete data set for all active compounds is included in Supplementary Material III).¹ Table 5 shows the results for the 18 compounds in Table 2. Among the 80 compounds tested, 17 exhibited toxicity as determined by the WST-1 assay, and 46 were positive by the toxicology multiplex, of which 28 were MGMT positive alone (Tables 5 and 6). Only two compounds, antimycin A and desmethylidihydrocapsaicin were WST-1 positive (reduced cell viability) and not active in the toxicology multiplex, whereas there were 29 compounds that were active in the multiplex but showed no effect on cell viability. Nine of the 17 WST-1-positive compounds showed significant reduction in total RNA yield. Further analysis indicates that there are 11 distinct expression patterns defined by the multiplex assay (Table 6). In all cases, the stress response genes were up-regulated in response to compounds, except for Act-D, which caused a reduction in mRNA levels for Hsp70, Gadd153,

Table 5 Toxicology multiplex analysis

Compounds were assayed in triplicate at 2 μM for 24, 36, and 48 h; gene expression change is the average fold change over this time period relative to vehicle control. WST-1 analysis (percentage change relative to vehicle control) for all compounds was for 24–48 h, except Act-D was for 36 h. -, no change.

Drug	Gene expression change					WST-1
	Hsp70	Gadd153	Gadd45	MGMT	Cyclo	
Acriflavium	-	-	-	-	-	-
Act-D	0.3	0.3	0.4	0	1.2	30
Aminopterin	1	1.9	1	0	1.1	17–39
Antimycin A	0.9	0.9	0.9	0	0.9	16–94
Chlortetracycline	0.9	0.7	0.9	4.6	0.9	-
Colchicine	-	-	-	-	-	-
Cycloheximide	1.1	1.1	1.7	0	1	42–55
Cytochalasin D	-	-	-	-	-	-
Digitoxin	1.2	2.6	1.9	0	1.1	12–39
Emetine	3.1	5.4	8.6	0	1.1	35–63
Gramicidin	0.7	6	2	0	0.8	58–86
Mitomycin C	1	3.2	3.2	0	0.9	9–44
Ouabain	1.1	2.9	2	0	0.9	20–43
Podophyllotoxin	-	-	-	-	-	-
Puromycin	1.1	5.1	1.8	3.1	0.8	10–28
Quinidine	-	-	-	-	-	-
Rotenone	0.8	0.8	0.8	3.4	1	0–88
Vinblastine sulfate	-	-	-	-	-	-

Table 6 Toxicology expression patterns

Summary analysis of Table 4. \uparrow is greater than 2-fold increase; - is no change; \downarrow is greater than 2-fold decrease.

Hsp70	Gadd153	Gadd45	MGMT	Cyclo	WST-1	Total
-	-	-	\uparrow	-	-	28
-	\uparrow	\uparrow	-	-	\uparrow	5
\uparrow	\uparrow	\uparrow	-	-	\uparrow	2
-	-	-	\uparrow	-	\uparrow	2
-	\uparrow	-	\uparrow	-	\uparrow	2
-	\uparrow	-	-	-	\uparrow	2
-	-	-	-	-	\uparrow	2
\uparrow	\uparrow	\uparrow	-	\uparrow	\uparrow	1
-	-	\uparrow	-	-	\uparrow	1
-	\uparrow	-	-	-	-	1
\downarrow	\downarrow	\downarrow	-	-	\uparrow	1

and Gadd45, the likely consequence of its direct inhibition of transcription. These results indicate that the toxicology multiplex is a more sensitive and specific indicator of potential toxicity than the WST-1 assay because compounds that cause, for example, DNA damage are sensitively detected by monitoring expression changes in stress response genes, whereas the WST-1 assay is unresponsive. Distinct expression profiles may be useful in defining drug classes with common toxicological properties.

Signaling Pathways and Gene Function. Drugs that have relatively well known effects on cellular signaling pathways can be used to evaluate the response of the gene targets to help characterize target function. We evaluated the effect of a select group of drugs on the response of the prostate target genes (Table 7). Survivin was the only gene whose expression was inhibited by the phorbol ester phorbol 12-myristate 13-acetate and the adenylyl cyclase activator forskolin, possibly by a mechanism result-

Table 7 Response of gene targets to selected drugs

+, stimulation; WST-1 is percentage inhibition relative to vehicle control. Drugs were assayed over a 2-log dose range in triplicate; the inhibition value (% I) is reported for the indicated drug concentration. PMA, phorbol 12-myristate 13-acetate.

Drug	% I	Drug concentration	Gene	WST-1
PMA	50	100 nM	Survivin	17
Forskolin	50	10 μM	Survivin	7
LY294002	50	10–100 μM	Survivin	30–63
	40	100 μM	hPSE	63
	+150	10–100 μM	HNF-3 α	30–63
RA	70	10 μM	Hoxb-13	-9
	50	10 μM	HNF-3 α	-9
β -Estradiol	20	1 μM	Hoxb-13	2
Hydrocortisone	35	1 μM	Hoxb-13	-3
Rapamycin	30	100 nM	Survivin	28
TSA	60–95	1 μM	All	27
Butyric acid	45–85	10 mM	All	9

ing in the reduction of mRNA stability because the survivin transcript is relatively stable in PC-3 cells (Fig. 3). A phosphatidylinositol 3'-kinase inhibitor, LY294002, inhibited survivin and hPSE, stimulated the expression of HNF-3 α over 2-fold, and exhibited high levels of toxicity. RA, a transcriptional activator, inhibited Hoxb-13 ($\text{IC}_{50} = 5 \mu\text{M}$) and HNF-3 α ($\text{IC}_{50} = 10 \mu\text{M}$), whereas steroid transcriptional activators estrogen and hydrocortisone and the immunosuppressive drug rapamycin had only weak effects on Hoxb-13 and survivin and no observable effect on the other target genes. TSA and butyric acid, compounds that cause global repression of histone deacetylase activity, block cell proliferation, and induce apoptosis, inhibited all of the prostate target genes, with butyric acid ($\text{IC}_{50} = 2\text{--}10 \text{ mM}$) exhibiting significantly lower toxicity than TSA.

Discussion

The results of this study validate MGE technology for high-throughput drug screening of multiple targets in parallel and demonstrate (a) the ability to identify drugs with distinct gene expression profiles, (b) reproducible determination of IC_{50} values for several classes of chemical compounds, (c) determination of structure-activity variation in a series of compounds comprising a single chemical class, (d) measurement of toxicological end points and the potential for providing early indication of compound toxicity in primary screening, and (e) elucidation of target gene responses to drugs affecting known signaling pathways.

For this study, the two multiplex assays consisted of 10 genes each. We have been successful in the development of MGE assays with multiplex sizes as high as 20 genes (data not shown). Gene expression analysis using the prostate target multiplex assay correlated well with two independent methods of gene expression analysis (TaqMan and Northern hybridization) as well as with activity measurements (*i.e.*, IC_{50} values) determined by completely different assay methods (as discussed below). The wide dynamic range of measured gene expression ratios relative to β -actin permits the determination of differences in gene expression of many orders of

magnitude that are not readily attainable using solid phase microarray techniques. Coupled with the larger dynamic range available from fluorescence readout technologies, it allows the simultaneous measurement of high and low copy transcripts. Although the CVs were somewhat high (18–34% CV), MGE analysis permitted the determination of changes in gene expression of 60% or greater with 95% confidence, allowing for the identification of relatively weak compounds. Confirmation of the initial “hits” and IC_{50} determinations was carried out on single compounds where intraplate reproducibility (9.5–14% CV) permitted determination of gene expression changes as low as 40% with 95% confidence. By measuring toxicological end points we identified compounds that affected DNA damage and stress response pathways that were not detected by a cell viability assay. The ratio of the spiked RNA control to an internal control, *e.g.*, β -actin, allows for the determination of RNA losses or degradation due to compound toxicity on dosed cells. Gene expression levels could be reproducibly determined even at toxic compound doses as measured by the WST-1 assay. Low total RNA requirements (10–30 ng) permit sample archiving for subsequent analysis based on typical RNA yields greater than 1 μ g/sample. Therefore, expression levels for hundreds of genes can be determined in cells from a single well of a 96-well plate. Throughputs can readily exceed 200 plates and 100 gene targets per week, generating over 250,000 data points, thus permitting rapid and economical drug profiling. Therefore, because MGE analysis can quantitatively analyze tens to hundreds of genes for tens of thousands of samples, it is capable of filling the technological gap between microarray hybridization analysis, useful for screening large numbers of genes but with low sample throughput, and real-time PCR, capable of high quantitative precision of a few genes over a few thousand samples.

Gene expression analysis using MGE technology provides a generic assay strategy for many types of drug targets including those of unknown function. The multiplex assay can identify both inhibitors and activators in different mechanistic classes (for example, compounds affecting transcription and mRNA stability). We also expect that the assay will identify compounds that affect self-regulating proteins and compounds that affect translation of the target gene protein and/or the protein function directly, as monitored by a downstream transcriptional readout(s) identified, for example, by hybridization array analysis of specific gene knockouts. The MGE assay provides quantitative data and can use multiple end points for defining specificity for chemical optimization.

MGE analysis can also measure toxicological end points in the primary screen to provide early indicators of drug safety. In this study, we identified compounds that affected DNA damage and stress response pathways that were not detected by a cell viability assay (WST-1 assay). Low total RNA requirements (10–30 ng) permit sample archiving for subsequent analysis based on typical RNA yields greater than 1 μ g/sample. Therefore, expression levels for hundreds of genes can be determined in cells from a single well of a 96-well plate. By comparing the results of Tables 2 and 5, one can readily identify potential toxicological properties (relating to DNA damage and cellular stress response) of

compounds identified as active in the prostate target screening assay. Nine of the 25 active compounds showed no toxicity effects, whereas 16 compounds showed defined toxicity profiles. Based on the known functions of the genes in the toxicology multiplex assay, one can infer some general information about the function of the prostate target genes that respond to particular compounds. For example, emetine and puromycin (two different classes of protein synthesis inhibitors) inhibit expression of the transcription factors Hoxb-13 and hPSE but cause a potent increase in the expression of Blx-33. Emetine, in particular, causes a pronounced activation of gadd45 as well as gadd153 and hsp70, genes, whose expressions are induced typically in response to growth arrest and DNA damage. These responses are p53 independent in the PC-3 cell line. Puromycin primarily affected gadd153 and was a much weaker inducer of Blx-33 than was emetine. The two compounds are further distinguished based on their MGMT responses. The function of Blx-33 has been implicated in apoptosis because its inhibition results in the activation of caspase 3 (data not shown). The activation of Blx-33 in response to compounds that induce expression of genes associated with DNA repair suggests that Blx-33 may function to block apoptosis in response to these drug treatments in a p53-negative cell line.

The MGE assay was used to analyze the kinetics of transcriptional inhibition by Act-D, a drug commonly used to inhibit mRNA synthesis and estimate mRNA stability (21). Differences in the IC_{50} values for the target genes may reflect the binding specificity of Act-D (22) for sequences in the promoter regions. The Act-D kinetic profiles for each of the target genes (Fig. 3) demonstrate the importance of evaluating the stability of target gene transcripts to use the most appropriate drug treatment time for screening compounds and to distinguish compounds that inhibit transcription from other types of mechanism, such as changes in mRNA stability. A 24-h drug treatment time represented a good compromise for high-throughput compound screening because mRNA half-lives were already obtained for most of the genes.

The finding that ouabain, digitoxin, and related compounds are potent inhibitors of several prostate target genes (Tables 2 and 4) suggests that the membrane Na^+K^+ -ATPase may directly affect transcriptional regulation of the prostate transcription factors Hoxb-13, hPSE, and HNF-3 α . The IC_{50} values determined for ouabain using the multiplex assay (29, 61, and 52 nM for Hoxb-13, hPSE, and HNF-3 α , respectively) are similar to the IC_{50} value for ouabain (27 nM) determined by an assay for enzyme ATPase activity using cardiac muscle cells (23). Important determinants for specific inhibition of the sodium pump include an unsaturated lactone moiety at position 17 and a hydroxyl group at position 14 (19). The results of analyzing over 40 cardiac glycosides suggest a SAR consistent with known information on this class of steroids as specific inhibitors of Na^+K^+ -ATPase (additional details and a more complete SAR analysis will be presented elsewhere). Digitoxin and ouabain have been suggested as potential anticancer drugs based on clinical studies and selective effects on normal *versus* tumor cells (24, 25).

The Na^+K^+ -ATPase is responsible for regulating Na^+K^+ exchange (26) and, as a consequence, intracellular Ca^{2+} levels involved in regulating the induction of apoptosis (6, 27–29). The β -1 subunit is under androgen regulation in PC-3 cells and has been implicated in prostate cancer (30). The finding that ouabain and digitoxin induce Gadd153 and Gadd45 (Table 5), genes involved in growth arrest and stress response, provides further insight into the mechanism of these drugs. Calcium, which is elevated in cells treated with cardiac glycosides (6), has been shown to play a role in the induction of Gadd153 mRNA expression through both an increase in transcriptional initiation and mRNA stabilization (31). Ectopic expression of Gadd153 can induce apoptosis in a p53-independent manner in leukemia cells (32). Etoposide, a topoisomerase II inhibitor, induces Gadd153 mRNA expression that occurs concomitantly with induction of apoptosis as measured by a DNA fragmentation assay (33). Other Ets transcription factors, related to hPSE, also play a role in regulating the expression of Gadd153 (34). Gadd45 expression causes G_2 arrest (35) and may be involved in p53-independent induction of apoptosis by BRCA1 (36). Furthermore, the demonstration that RA inhibits the transcription of Hoxb-13 and HNF-3 α (Table 6) provides an additional link between these two transcription factors and the apoptosis-inducing effects of bufalins (Table 4) and suggests that Hoxb-13 and HNF-3 α are involved in RA-regulated pathways. It is interesting to note that RA enhances the functional and morphological differentiation effects of bufalin in primary acute promyelocytic leukemia cells (37), whereas Gadd45 has been shown to bind to RA receptors and act as a nuclear coactivator (38).

We have discovered that the inhibition of Na^+K^+ -ATPase, which selectively induces apoptosis in PC-3 cells but not BPH-1 cells, also results in inhibition of the expression of survivin (Table 4). Survivin is a member of the inhibitor of apoptosis protein family whose down-regulation has been shown to increase apoptosis and inhibit cytokinesis (16, 39). Survivin associates with the mitotic spindle and is involved in inhibiting caspase activity. Survivin is not expressed in differentiated adult tissues but is expressed in a variety of human tumors (15). In HeLa cells, it is expressed in the G_2 -M phase of the cell cycle in a cell cycle-regulated manner. Survivin is a p53-repressed gene (40) that, in a p53-negative cell line such as PC-3, is consistent with a role in the induction of apoptosis after its down-regulation through inhibition of Na^+K^+ -ATPase. We note also that Act-D induced or inhibited survivin mRNA depending on drug concentration and time (Fig. 3), suggesting that regulation of survivin mRNA synthesis and stability in PC-3 cells is complex.

Evaluating the effects of known drugs on target genes of relatively unknown function can provide information linking the target genes to known cellular pathways and drug mechanisms. For example, RA was discovered to selectively inhibit Hoxb-13 and HNF-3 α (Table 7), two transcription factors overexpressed in prostate cancer whose inhibition by specific antisense reagents results in inhibition of PC-3 cell proliferation (data not shown). The effects of RA are mediated by two classes of nuclear proteins (41), ligand-regulated transcription factors regulating cell proliferation, differentia-

tion, and apoptosis. RA induces tissue transglutaminase, a Ca^{2+} -dependent enzyme, and Bcl-2, two proteins involved in apoptosis regulation (42, 43). RA inhibition of Hoxb-13 and HNF-3 α suggests that these two transcription factors are involved in RA-regulated pathways. It is interesting to note that RA enhances the functional and morphological differentiation effects of bufalin in primary acute promyelocytic leukemia cells (37). The demonstration that RA inhibits the transcription of Hoxb-13 and HNF-3 α provides a further link between these two transcription factors and the apoptosis-inducing effects of bufalins. Furthermore, Gadd45 has been shown to bind to RA receptors and act as a nuclear coactivator (38).

Recent studies have demonstrated that a large number of genomic alterations in a cancer cell appear early in sporadic tumor development (44, 45). Genomic instability, a characteristic of virtually all types of cancers, results in hundreds of genes being differentially expressed between the normal and cancer cell (46). We have recently shown that gene expression profiles can predict the aggressive behavior of breast cancer cells and constitute a molecular phenotype for invasive carcinomas (4). Because many genes contribute to the complex set of altered cellular interactions that comprise the disease state, it is unlikely that a single target plays a dominant mechanistic role in a complex disease such as cancer. Multiparameter cell-based assays such as the MGE assay described here will facilitate screening for the complex gene expression changes that characterize a disease state and that collectively may represent the most effective drug target.

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