Nonspecific enhancement of gene expression by compounds identified in high-throughput cell-based screening

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Cell-based reporter assays are increasingly used for high-throughput screening of chemical libraries. However, reporter plasmids are subject to artifacts arising from any source of variance in promoter activity. For example, some common vectors have been found to contain occult steroid- or transcription-factor-responsive elements (1–4). Because reporter plasmids are frequently used in molecular and cellular biology to report pathway activity after treatment with experimental agents, it would be helpful for investigators to be aware of such effects, how often they occur, in which classes of structures they are often observed, in which direction the activity is likely to shift, and how subtle or robust is the artifact. In addition, nontranscriptional artifacts of compound screening include contaminants that reflect or absorb light in colorimetric assays, autofluorescence of cells, plastics, and media that decreases the signal-to-noise ratio in fluorometric

assays and decreases the aggregates of small molecules that serve as inhibitors of cell-surface receptors (5).

We recently reported the novel histone deacetylase inhibitor (HDACI) Scriptaid, which was identified through screening of a compound library on a Smad4-reporter (Smad4-R) cell line (6). Scriptaid, like trichostatin-A (TSA) (7), is a hydroxamate that nonspecifically up-regulates transcription of multiple genes by inhibiting the deacetylation of lysine residues on histones. Here, we extend our screening experiments to explore additional agents, found among diverse compounds and among drugs currently in clinical use, that share the characteristic of indiscriminately augmenting gene expression.

We screened a 16,320-compound library (DIVERSet E; ChemBridge, San Diego, CA, USA) and an 880-drug library (i.e., agents previously tested for acceptable bioavailability and toxicity profiles: Monotarget Set; Prestwick

Chemical, Washington, DC, USA). Each compound in the screen was diluted in dimethylsulfoxide (DMSO) at 0.5 mg/mL (compounds) or 1 mg/mL (drugs). Cells containing transcriptional reporter systems (Table 1) were plated, incubated with each compound at 2 µg/mL, and tested as described (6).

The 17,220 agents (16,320 compounds and 880 drugs) were screened initially against Smad4-R cells and p53-reporter (p53-R) RKO cells (8). There were 14 agents (structures shown in supplementary Figure S1, found on the BioTechniques web site at <http://www.BioTechniques.com/July2004/CunninghamSupplementary.html>) that activated both Smad4-R and p53-R cell lines more than 2-fold and were therefore chosen as a panel to test on six other available reporter plasmid systems over a concentration range of 1 to 10 µg/mL. Seven compounds (0.04%) and five drugs (0.57%) indiscriminately elevated the activity of multiple reporters; one of the compounds was Scriptaid. All reporter systems were up-regulated upon treatment except MDA-kb2, containing the mouse mammary tumor virus (MMTV) promoter, which is known to be repressed by HDACIs (9). Eleven agents activated two or more reporter systems; eight activated three or more (Table 2) (activation was defined as a greater-than two-fold induction). This effect was independent of type of reporter [e.g., luciferase-based versus fluorescence-based (Table 1)] and the type of transfection [transient versus stable (data from Ref-

Table 1. Plasmid Reporter Systems

Cell Type	References	Reporter Name	Reported	Emission	Promoter	Enhancer
HPC (PANC-1)	6	Smad4-R	Smad4 function	FF-luc	min SV40	6SBE
HCC (RKO)	8	p53-R	p53 binding	FF-luc	min SV41	
HCC (HCT116)	11	p53-R	p53 binding	FF-luc		
HCC (DLD-1)	12	DLD/BFP	constitutive	Fluor	CMV	
CHO	13	CHOAA8-luc-tet-off	constitutive	FF-luc	CMV	
MEF (NIH/3T3)	14	Shh Light 2	Hedgehog protein	FF-luc		
MEF (NIH/3T3)	14	Shh Light 2	constitutive	Ren-luc	HSV-TK	
HBC (MDA-kb-2)	15	MDA-kb2	AR, GR stimulation	FF-luc	MMTV	

Eight different reporter plasmids stably transfected into seven cell lines were used to test a panel of agents that activated the Smad4-reporter system. CHO AA8 Tet-Off cell line was obtained from BD Biosciences Clontech, San Jose, CA, USA. MDA-kb2 cell line was obtained from ATCC, Manassas, VA, USA. Other cell lines were gifts as described in the Acknowledgments section or engineered in our laboratory as referenced.

Abbreviations: HPC, human pancreas carcinoma; HCC, human colon carcinoma; MEF, mouse embryonal fibroblasts; HBC, human breast carcinoma; CHO, Chinese hamster ovary; AR: androgen receptor; GR: glucocorticoid receptor; SV40, simian virus 40; CMV, cytomegalovirus; HSV-TK, herpes simplex virus thymidine kinase; MMTV, mouse mammary tumor virus.

Table 2: Fold Activation Over DMSO Control

Agent Tested	Reporters Activated (No.)	Reporter Systems						
		1 Smad4R	2 RKO p53R	3 HCT116 p53R	4 DLD/BFP	5 CHO-AA8	6 Shh FF	7 Shh REN
Quinacrine	6	4.1 @ 2	18.9 @ 2	16.7 @ 5	1.3 @ 5	23.1 @ 10	14.5 @ 5	2.4 @ 1
PTT	3	1.8 @ 5	2.2 @ 2	1.9 @ 5	1.5 @ 5	10.8 @ 10	4.2 @ 5	1.4 @ 1
Piperine	2	1.7 @ 5	1.8 @ 1	1.9 @ 10	1.4 @ 5	4.2 @ 10	1.8 @ 1	3.1 @ 1
Apigenin	4	1.6 @ 2	2.6 @ 5	2.4 @ 10	1.2 @ 1	4.6 @ 10	1.6 @ 1	2.5 @ 1
Hydralazine	1	1.7 @ 5	1.6 @ 2	1.6 @ 10	1.4 @ 5	1.4 @ 10	1.6 @ 1	2.3 @ 1
5110235	1	1.7 @ 5	1.8 @ 2	1.8 @ 10	1.4 @ 2	3.3 @ 10	1.8 @ 1	2.2 @ 1
5100018	2	1.0 @ 1	3.6 @ 1	1.0 @ 1	1.6 @ 5	1.4 @ 10	1.4 @ 1	3.6 @ 2
5175328	3	1.2 @ 1	3.1 @ 1	17.1 @ 2	1.3 @ 1	27.3 @ 1	9.4 @ 1	1.2 @ 1
5175323	5	4.2 @ 5	13.0 @ 5	11.4 @ 10	1.5 @ 10	9.7 @ 10	5.5 @ 10	2.2 @ 1
5145324	7	3.3 @ 1	5.2 @ 1	12.5 @ 2	3.4 @ 10	25.9 @ 2	9.2 @ 2	2.9 @ 1
5234881	3	1.2 @ 1	1.5 @ 1	1.8 @ 1	1.4 @ 5	2.7 @ 1	4.5 @ 2	2.2 @ 1
5238219	4	1.8 @ 5	1.9 @ 2	1.8 @ 2	2.0 @ 10	11.9 @ 10	3.4 @ 5	2.0 @ 2
5268029	0	1.4 @ 10	1.7 @ 10	1.3 @ 10	1.3 @ 2	1.3 @ 10	1.3 @ 1	1.7 @ 1
Average SEM/mean		0.036	0.064	0.027	0.016	0.039	0.047	0.041
Formal names of agents tested: Quinacrine, quinacrine dihydrochloride dihydrate; PTT, 1-phenyl-3-(2-thiazolyl)-2-thiourea; Piperine, piperine; Apigenin, apigenin; Hydralazine, hydralazine hydrochloride; 5110235: 2-methyl-4-phenyl-pyrido[1,2-a]pyrimidin-5-ylum (perchlorate); 5100018, 5-benzenesulfonylamino-2-methyl-1-phenyl-1H-indole-3-carboxylic acid ethyl ester; 5175328, acridin-9-yl-[4-(4-methyl-piperazin-1-yl)-phenyl]-amine; 5175323, N4-benzo[g]quinolin-4-yl-N1,N1-diethyl-pentane-1,4-diamine; 5175324, benzo[g]quinolin-4-yl-(1-ethyl-piperidin-3-yl)-amine; 5234881, 4-[(4-benzothiazol-2-yl-phenylimino)-methyl]-phenol; 5238219, 3-(2-hydrazono-2-p-tolyl-ethylidene)-3,4-dihydro-1H-quinoxalin-2-one; 5268029, 5-(4-Hydroxy-benzylidene)-3-(4-hydroxy-phenyl)-2-thioxo-thiazolidin-4-one. Activation defined as >2-fold increase in reporter activity. Highest-fold induction of reporter activity (compared with DMSO) @ dose used to effect highest-fold induction (µg/mL). All experiments were performed in triplicate, and the experiments were replicated on two to four independent occasions with similar results. The average SEM/mean for all drugs is indicated, with 98% of the values typically falling between 0.004 and 0.13. DMSO, dimethylsulfoxide.								

erence 6)]. Four of the 11 agents (36%) were structurally similar (quinacrine, 5175323, 5175324, and 5175328 all share a similar heterotricyclic functional group that is known to intercalate into DNA), and these generally produced the largest induction of the reporter systems (Table 2). All assays were performed in triplicate, and all experiments were replicated independently on two to four separate study periods.

Unlike TSA and Scriptaid, this group of agents did not appear to have histone deacetylation as the mechanism of action, as evidenced by the relatively stable level of acetylated histones as assayed by immunoblot (data not shown). Some compounds produced minor changes in levels of methylhistone H3 and no apparent changes in phospho-histones (data not shown). Although a systematic evaluation of the mechanism

of the agents' described effects was not undertaken, non-HDACI possibilities for nonspecifically facilitating expression include augmenting the activity of ubiquitous occult enhancer elements, DNA polymerases, RNA polymerase, mRNA-stabilizing proteins, and poly-somes or by suppressing proteasome-dependent proteolysis. We suspected that these agents would synergize with TSA due to a differing mechanism of action, and indeed several of them did.

To demonstrate this, we measured light emission from p53-R RKO cells after incubation with each test agent and escalating doses of TSA. The test agents were two compounds (5100018 or 5175323) or two drugs (quinacrine or apigenin) at a single dose. Varying doses of TSA with or without the single dose of compound were added to cells to construct a dose scale (0, 0.05, 0.1,

0.2, 0.4, or 0.8 µg/mL TSA). A merely additive effect was observed when 5100018 was combined with TSA. For example, 2 µg/mL 5100018 added to a given dose of TSA produced a similar increase as did an equipotent additional dose of TSA when tested at multiple points in the TSA dose scale. A robustly synergistic effect was seen however, when 5175323, quinacrine, and apigenin were added to TSA, again testing at multiple points along the scale. For example, a doubling of TSA alone from 0.05 to 0.1 µg/mL increased luciferase activity by approximately 100%, but the addition of an equipotent dose of 5175323 to 0.05 µg/mL TSA resulted in an increase in luciferase activity by about 700%, a 7-fold increase that is evidence of a synergistic relationship. Similar synergistic effects were produced by quinacrine and apigenin. Syn-

Figure 1. Response of the *Renilla Shh-Light2* reporter cells to a panel of expression facilitators. Data points represent the average of the readings of triplicate wells of a representative experiment. The average of the SEM/mean for all points was 0.041. TSA, trichostatin-A; PTT, 1-phenyl-3-(2-thiazolyl)-2-thiourea.

ergy was maintained in the presence of 4 mM caffeine for p53-R cells and replicated in the Smad4-R cells (data not shown), excluding p53 pathway activation (due to DNA damage) as an artifactual mimic of synergy. All synergy assays were performed in duplicate, and experiments were replicated on two or three separate study periods.

Some agents had optimal reporter effects at a concentration that was borderline toxic to cells, especially true of quinacrine and TSA (Figure 1) and of 5175323 and 5175324 in other reporter systems (data not shown). Although some transcriptional facilitators [e.g., the HDACI suberoylanilide hydroxamic acid (SAHA)] are already in clinical trials (10), the observation that expression facilitation is seen at near toxic levels generally reflects the established concern for the safety of the classes of compounds that would be expected nonspecifically to up-regulate transcription in patients. Other compounds (e.g., 5175328 in Figure 1) acted only to reduce reporter activity, and most of such compounds are presumed to be toxic.

Compound and drug libraries are commonly screened in search of novel therapeutics. Compound libraries

can be expected to contain a group of agents nonspecifically modulating gene expression. This expectation will affect the planning and interpretation of such endeavors.

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