

A Novel Histone Deacetylase Inhibitor Identified by High-Throughput Transcriptional Screening of a Compound Library¹

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Abstract

Libraries of compounds are increasingly becoming commercially available for the use of individual academic laboratories. A high-throughput system based on a stably integrated transcriptional reporter was used to screen a library of random compounds to identify agents that conferred robust augmentation of a signal transduction pathway. A novel histone deacetylase (HDAC) inhibitor, termed scriptaid, conferred the greatest effect, a 12- to 18-fold augmentation. This facilitation of transcriptional events was generally applicable to exogenous gene constructs, including viral and cellular promoters, different cell lines and reporter genes, and stably integrated and transiently introduced sequences. Scriptaid did not interfere with a further induction provided by stimulation of the cognate signal transduction pathway (transforming growth factor β /Smad4), which implied the functional independence of ligand-stimulated transcriptional activation and histone acetylation states in this system. Additional insights into this and other signal transduction systems are likely to be afforded through the application of compound screening technologies.

Introduction

Many signal transduction pathways couple ligand-initiated events that affect the regulation of gene expression. The elaboration of signal transduction pathways is important in tumor biology because the mediators and regulators of signaling pathways can represent useful targets for diagnosis and therapy.

Currently, the common methods used to identify the mediator and regulators of a pathway involve physical interactions (two-hybrid analysis and protein affinity chromatography), experimental genetic means (mutagenesis of bacteria or yeast), and observations of natural genetic variance (human tumors and inherited disease susceptibility). Cloning by physical interactions is rather inefficient and laborious and can be biased by the initial choice of the bait. Experimental genetic methods are efficient and unbiased but difficult to apply to mammalian cells and human disease. Research based on natural genetic variance is highly relevant to the understanding of human disease, but the sample size of such an approach is often limited. The screening of random libraries of chemical compounds can encompass all of the benefits of current methodologies; they can be unbiased and high-throughput and can be used to probe and dissect complex biological systems in mammalian cells (1, 2). Screening of compound libraries can also directly produce candidates for therapeutic and experimental applications.

The “true” activation of a signaling or regulatory pathway can be difficult to measure. The detection of a downstream transcriptional

event using a specific reporter construct in cells is a common method. This type of manipulation of a pathway requires a basic understanding of the participating members of the pathway, but the understanding is often incomplete and imprecise. To test a promoter or an enhancer under adequate sensitivity, the selection of reporter genes often is limited. The interpretation of results using downstream reporter constructs can be misled by unanticipated positive or negative influences. That is, it is often difficult to distinguish release of inhibition from true activation, and *vice versa*. Furthermore, the system depends on the recruitment of the general transcriptional apparatus, the interaction of which with the system under study is seldom known in advance.

We chose to address these issues by the implementation of a high-throughput compound screening, using a stably integrated reporter construct to identify reliable and important regulators of a tumor-suppressive pathway. The TGF β ³ pathway is well studied biologically and comprises a number of human tumor-suppressor genes, including *SMAD4* (*MADH4*, *DPC4*; Refs. 3–5). Our reporter construct contains Smad-binding elements (p6SBE-luc) and allows us to measure processes that result in the nuclear localization of Smad4 (6). The discovery of agents that would interact with or bypass deficits in the TGF β pathway by augmenting the action of downstream Smad4 would likely be useful to the biological understanding of this tumor-suppressive pathway.

Materials and Methods

Reporter Constructs. p6SBE-luc and p6MBE-luc were engineered by inserting six copies of the palindromic SBE or of the MBE (an inactive mutant version) behind the minimal SV40 promoter in the pGL3-promoter vector (Promega, Madison, WI; Ref. 6).

Cell Lines. PANC-1 and MDA-MB-468 cell lines were purchased from American Type Culture Collection (Manassas, VA). Stable transfectants were generated by cotransfection of pcDNA3.1 (Invitrogen, Carlsbad, CA) and p6SBE-luc into PANC-1 cells with lipofectamine (Life Technologies, Inc.). Transfected cells were diluted and selected in multiple 96-well plates in the presence of 0.5 mg/ml G418 (Life Technologies, Inc.). Single clones were expanded and tested for basal luciferase expression and TGF β inducibility. One clone was chosen on the basis of high (6- to 8-fold) induction of luciferase by 0.5 ng/ml TGF β (R&D Systems, Minneapolis, MN).

Compound Screening. Each compound of the library (DIVERSet, Chem-Bridge, San Diego, CA) was dissolved and diluted in DMSO at 1 mg/ml. Cells were plated in 96-well cluster plates (Corning, Cambridge, MA) and incubated with each compound, after further dilution in culture medium to the final concentration of 2 μ g/ml, for 16–18 h. Luciferase activity was measured on the addition of Steady-Glo substrate (Promega). Up to 16 96-well plates could be assembled in a Wallac Trilux photodetector (Wallac, Gaithersburg, MD) for measurement. All of the readouts from each experiment were compared with the control wells, and a number reflecting the relative increase in luciferase activity was calculated for each chemical by using Excel (Microsoft, Redmond, WA) spreadsheets.

³ The abbreviations used are: TGF, transforming growth factor; HDAC, histone deacetylase; TSA, trichostatin A; SBE, Smad-binding element; MBE, mutated SBE; β -gal, β -galactosidase; CMV, cytomegalovirus; CBP, CREB-binding protein.

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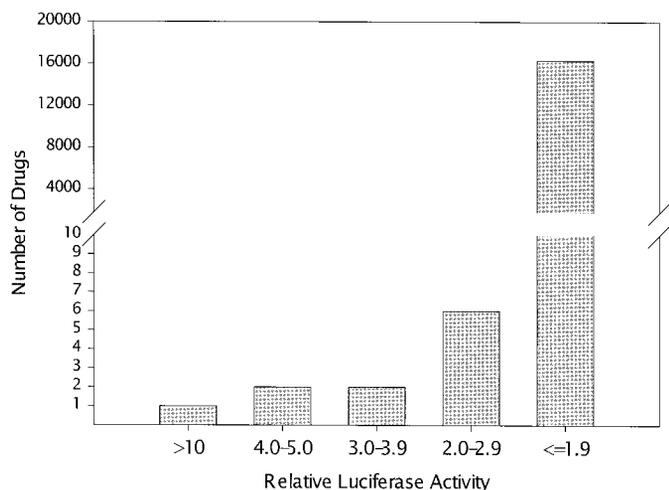


Fig. 1. The distribution of compounds in accordance to their relative luciferase activity. The entire library, consisting of 16,320 compounds, was screened with the p6SBE-luc reporter construct stably integrated in PANC-1 cells. The relative luciferase activity was calculated in comparison with untreated cells.

Immunoblotting Assay of Histone Acetylation. PANC-1 cells were treated with 2 $\mu\text{g}/\text{ml}$ of scriptaid (ChemBridge) or 0.1 to 0.32 $\mu\text{g}/\text{ml}$ of TSA (Sigma, St. Louis, MO) for 18 h in culture medium. Treated and untreated cells were harvested with trypsin-EDTA (Life Technologies, Inc.), washed with PBS (Life Technologies, Inc.), and resuspended in a protein sample buffer. Protein concentration was determined by BCA protein assay reagents (Pierce, Rockford, IL). Fifty μg of proteins from each sample was loaded on a 12% denaturing polyacrylamide gel. Proteins were subsequently transferred to a nylon membrane (Imobilon P, Millipore, Burlington, MA) using Milliblot-Graphite Electrobloetter I (Millipore). The nylon membrane was incubated with rabbit antihuman acetyl-lysine antibody (Upstate Biotechnology, Waltham, MA) at 1:1000 dilution, followed by goat antirabbit antibody coupled to horseradish peroxidase (Pierce) at 1:2000 dilution, developed with SuperSignal substrates (Pierce), and detected by film (BioMax, Kodak, Rochester, NY).

Survival Curve. Equal numbers of cells were plated in six-well plates in the absence or presence of scriptaid or TSA at different concentrations. After 18 h of incubation, cell numbers were determined by trypan blue exclusion. Percent survival of the treated cells was calculated in comparison to the untreated sample, which was considered to represent 100%.

Transfection Assay. Each transient transfection experiment was done in duplicate in six-well plates. Lipofectamin (Life Technologies, Inc.) was used as directed by the manufacturer. The DNA-lipofectamin mixture was removed from cells after 4–5 h of transfection, and culture media with or without compounds or TGF β was then added to the cells. Sixteen to 18 h from the start of the transfection, cell lysates were prepared with Reporter Lysis Buffer (Promega) for luciferase and β -gal assays. Luciferase was measured using The Luciferase Assay System (Promega) and β -gal assay was performed as described previously (6). Studies of the SV40 promoter included all of the experiments performed with p6SBE-luc, p6MBE-luc, and pGL3-control (Promega) plasmids. Studies of the CMV promoter were done using pCMV β (Clontech, Palo Alto, CA), and those of human ubiquitin c promoter were done using pUB6/V5-lacZ (Invitrogen).

Results

Identification of Scriptaid. The entire library, consisting of 16,320 compounds, was screened. Eleven compounds were associated with a 2- to 5-fold induction of luciferase activity, and one with a 12-fold activation (Fig. 1). Additional studies on the latter compound (Identification No. 217444; 6-(1,3-dioxo-1*H*,3*H*-benzo[*de*]isoquinolin-2-yl)-hexanoic acid hydroxyamide, we termed scriptaid) are reported here (Fig. 2). A related compound (Identification No. 158497; 4-(1,3-dioxo-1*H*,3*H*-benzo[*de*]isoquinolin-2-yl)-*N*-hydroxy-butyr- amide, we termed nullscript), which did not induce the p6SBE-luc reporter construct in the initial screen, was identified from the

library using ChemFinder (Cambridge Soft, Cambridge, MA) by its structural similarity to scriptaid (Fig. 2). The results were validated by repeated determinations in the screening assay and subsequently by a dose-response curve performed on PANC-1 cells that contained stably integrated p6SBE-luc (Fig. 3).

Scriptaid As a Novel Inhibitor of HDAC. Scriptaid was speculated to be a novel HDAC inhibitor because of its structural similarity to the class of hydroxamic acid-containing HDAC inhibitors, which

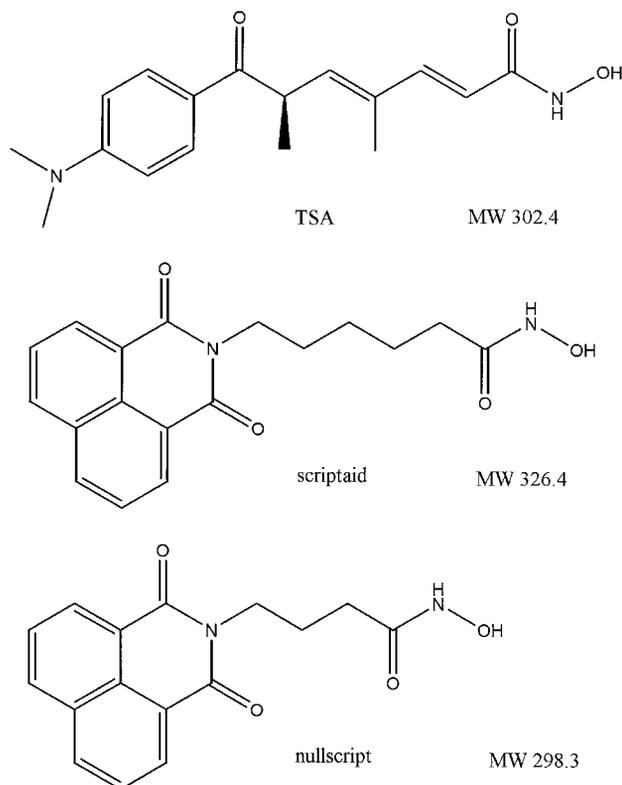


Fig. 2. Structural similarities of TSA, scriptaid, and nullscript. TSA, scriptaid, and nullscript possess the same hydroxamic acid group, an aliphatic chain, and an aromatic cap at the other end. The aliphatic linkers of TSA and scriptaid are 5-carbon in length, whereas the linker is only 3-carbon in nullscript.

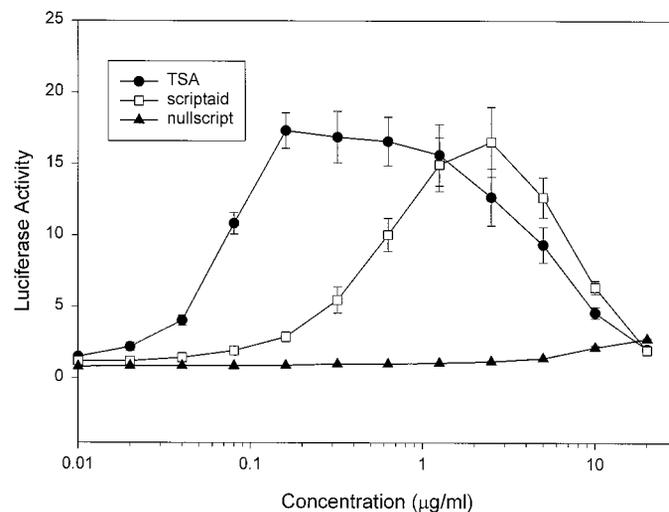


Fig. 3. Dose responses of TSA and scriptaid transcriptional facilitation in a stably transfected cell line. Luciferase activity was determined using PANC-1 cells having stably integrated p6SBE-luc at the indicated concentration of compounds. Data represent averages of two to three experiments and SE.

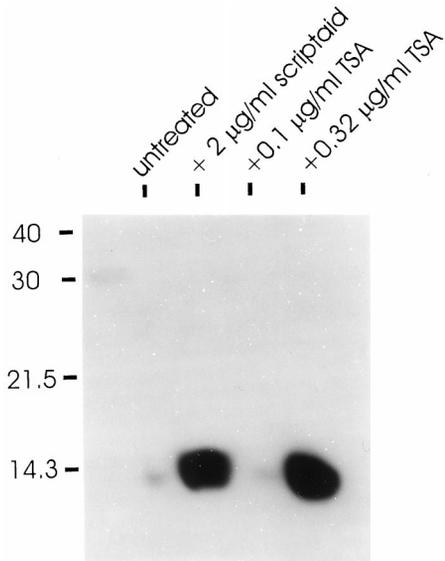


Fig. 4. HDAC inhibition by scriptaid. PANC-1 cells were untreated or treated with scriptaid (2 $\mu\text{g/ml}$) or TSA (0.1 or 0.32 $\mu\text{g/ml}$). Acetylated histones were detected by antihuman acetyl-lysine antibody immunoblot.

include TSA (Fig. 2). The direct interaction of TSA and a HDAC has been examined by crystallography (7). The hydroxamic acid group of TSA coordinates the zinc atom in the polar pocket of HDAC in the crystal structure of the HDAC-Zn²⁺-TSA complex. The hydroxamic acid group on TSA is attached to a five-carbon (excluding the carbon elements of the hydroxamic acid group or the keto group) aliphatic chain that spans a narrow tube-like pit formed by the surface of HDAC. The double bonds and the methyl-group in the aliphatic chain of TSA (Fig. 2) are not necessary for its inhibitory function (7). The bulky end-group on the opposite end of the aliphatic chain is positioned outside the entrance to the pit. Similar to TSA, scriptaid has a five-carbon linker between a bulky end-group and the hydroxamic acid moiety. Nullscript, which is almost identical to scriptaid except for a three-carbon (rather than five-carbon) linker (Fig. 2), was inactive in transcriptional facilitation at corresponding concentrations (Fig. 3), which confirmed a minimal requirement for the length of the linker chain expected for this class of HDAC inhibitors.

The use of scriptaid resulted in a >100-fold increase in histone acetylation (Fig. 4) in cultured cells, which confirmed scriptaid as a HDAC inhibitor.

Functional Comparison with TSA. To evaluate the potency of scriptaid, TSA was used as the reference compound in the following experiments. Optimal concentration was determined for both scriptaid and TSA (Fig. 3). Scriptaid worked optimally at 2–2.5 $\mu\text{g/ml}$ (6–8 μM), and TSA activity peaked at 0.32 $\mu\text{g/ml}$ (1 μM). At its optimal concentration for transcriptional facilitation, scriptaid was not lethal to one cell line and had limited effects (80% survival) on another (Fig. 5A). TSA was cytotoxic for two cell lines at its optimal concentration range (Fig. 5B). TSA at its minimal toxic concentration (0.1 $\mu\text{g/ml}$; 85–90% survival) was less efficient at inhibiting endogenous histone deacetylation (Fig. 4).

Smad proteins can physically associate with the histone acetylases p300 or CBP, and it has been suggested that the TGF β /Smad4 signaling pathway might activate gene transcription through such a mechanism (8–10). It was, therefore, important to determine whether the manipulation of histone acetylation status would interfere with the results of an assay for relative transcriptional induction mediated by the SBE sequence. It has been shown previously that p6SBE-luc, but not p6MBE-luc, can be induced by TGF β treatment (6). pCMV β was,

therefore, cotransfected with p6SBE-luc or p6MBE-luc as a TGF β -insensitive control. The presence of scriptaid increased the transcription of all of the three reporters by twelve-fold. Using a normalization for (cotransfected) β -gal expression, the measured magnitude of the ability of TGF β to specifically induce p6SBE-luc remained the same (Fig. 6A), irrespective of the presence or absence of scriptaid. Scriptaid could thus facilitate transcription independent of a positive inducer of transcription, producing multiplicative rises in reporter activity. Similar effects were seen with either scriptaid or TSA when the reporter construct was stably integrated into the host cell genome (Fig. 6, B and C). Scriptaid and TSA can proportionally enhance the induction of an integrated p6SBE-luc construct without interfering with TGF β -stimulated transcriptional responses.

To further evaluate the range of promoter elements subject to scriptaid induction, additional reporters were used in transient transfections. Scriptaid was capable of inducing high expression of p6MBE-luc (Fig. 7A), pCMV β (Fig. 7B), and pUB6/V5-LacZ (Fig. 7C), driven by viral (SV40 and CMV) or human (ubiquitin c, UB6) promoters. This general facilitation of transcription by scriptaid did not depend on the specificity of the enhancer (SBE *versus* MBE), the type of promoter (viral *versus* cellular), the product of the reporter gene (luciferase *versus* β -gal), nor on the integration status of the

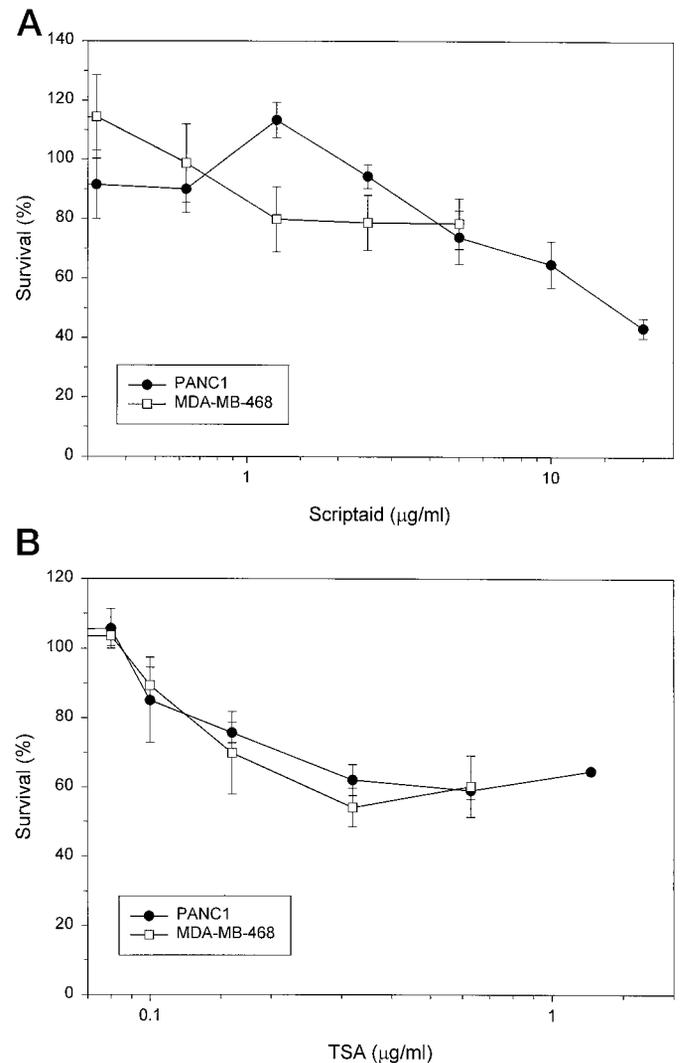


Fig. 5. Effects of compounds on cell survival. The survival of PANC-1 and MDA-MB-468 in the presence of scriptaid (A) and TSA (B) was determined by trypan blue exclusion after an 18-h incubation in the presence of compound. Data represent averages of two to four experiments and SE.

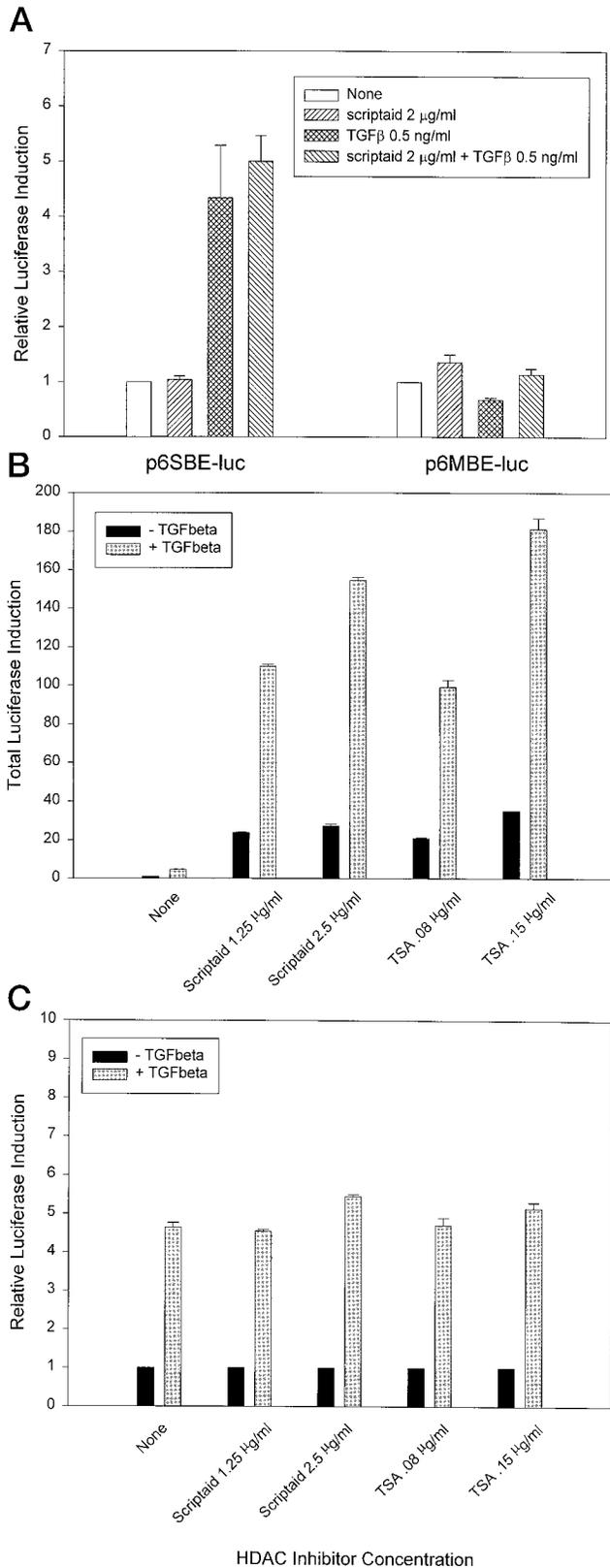


Fig. 6. Proportional effects of scriptaid and TSA on the TGF β /Smad4 signal transduction assay. *A*, p6SBE-luc or p6MBE-luc was cotransfected with pCMV- β into PANC-1 cells. Transfected cells were untreated or treated with TGF β or/and scriptaid for 18 h. Luciferase activities were proportionally enhanced in the presence of scriptaid. Relative luciferase induction was determined after normalization to the TGF β -noninducible pCMV- β control, itself subject to scriptaid facilitation (see Fig. 7). Data represent averages of two experiments and SE. *B* and *C*, PANC-1 cells containing the stably integrated p6SBE-luc were treated with scriptaid or TSA in the absence or presence of TGF β (1 ng/ml). Total luciferase induction is presented in *B*. Relative luciferase induction (*C*) was determined after normalization to the values observed with the HDAC inhibitor alone.

reporter construct (stable *versus* transient). The ability of scriptaid to facilitate transcriptional activation was consistently robust and concentration-dependent in both stable and transient reporter assays (Fig. 3, 7, and data not shown). In contrast, the performance of TSA was less predictable at its optimal concentration (Fig. 7, *A* and *B*; see *MDA-MB-468*). Lowering the concentration of TSA (0.1 $\mu\text{g/ml}$) could mitigate this lack of consistency, but in doing so, the efficiency of TSA as an HDAC inhibitor or a general transcription facilitator was significantly compromised (Fig. 4 and 7).

Discussion

We successfully confirmed the feasibility of a high-throughput reporter system to efficiently screen a large compound library in mammalian cells. The screening identified several compounds capable of augmenting the reporter activity, and a specific mechanism for one of the compounds was defined. This confirmed the suggested utility of this application of compound screening in mammalian cells for the discovery of additional small compound interactors that could help characterize the components of this pathway.

Scriptaid is a novel HDAC inhibitor that belongs to an existing class of hydroxamic acid-containing HDAC inhibitors. Scriptaid possesses a general property of transcriptional facilitation that applies to stably integrated or transiently transfected exogenous constructs, to promoters derived from viruses or an endogenous gene, to multiple reporter genes, and to different cell lines. Scriptaid does not interfere with the ability of a reporter construct to measure the positive (purely inductive) activation of a transcription factor in response to a known signal transduction stimulus. In relation to other members of its class, the optimal concentration of scriptaid (6–8 μM) is similar to those reported for suberoylanilide hydroxamic acid (2 μM) and *m*-carboxycinnamic bis-hydroxamide (4 μM ; Ref. 11), higher than TSA (1 μM , as measured here), and much lower than those reported for hexamethylenbisacetamide (5000 μM) and diethyl bis-(penta-methylene-*N,N*-dimethylcarboxamide) malonate (400 μM ; Ref. 11). Our data suggested some advantages of scriptaid over TSA in the range of promoters subject to predictable effects (Fig. 4 and 7) and in cellular toxicity (Fig. 4 and 5), although some degree of cellular toxicity may be a general feature of this class of compounds when used at transcriptionally effective concentrations (11).

The ability of scriptaid to indiscriminately facilitate transcriptional activation and its facilitation of detection of a positive transcriptional signal suggest the usage of scriptaid as a useful reagent for transactivation assays in reporter systems, perhaps allowing the use of difficult-to-transfect cells, the use of the available but less sensitive reporter genes, such as green fluorescent protein, or the minimization of culture volumes to aid high-throughput compound or biological screening and for adaptation to robotic handling. A reduction in the signal transduction strength needed to detect the operation of a reporter suggests a utility in the measurement of signal transduction events at a lower and thus more physiological range. For example, the use of scriptaid would be expected to reduce the requirement for protein overexpression or for high (pharmacological) levels of ligand often used to facilitate the evaluation of a signaling pathway. Application to other protein expression methods is also possible.

Currently underappreciated is the strength of the background of transcriptional repression that acts on general-utility promoters. Use of a relative nontoxic HDAC inhibitor such as scriptaid, thus, could conceivably simplify the interpretation of transcriptional reporter assays. We observed that at least 90% of the potential magnitude of the inducible transcriptional activation of our reporter system was originally repressed. It is known that the expression of some genes is regulated by the degree of histone acetylation (12). Thus, a 2-fold

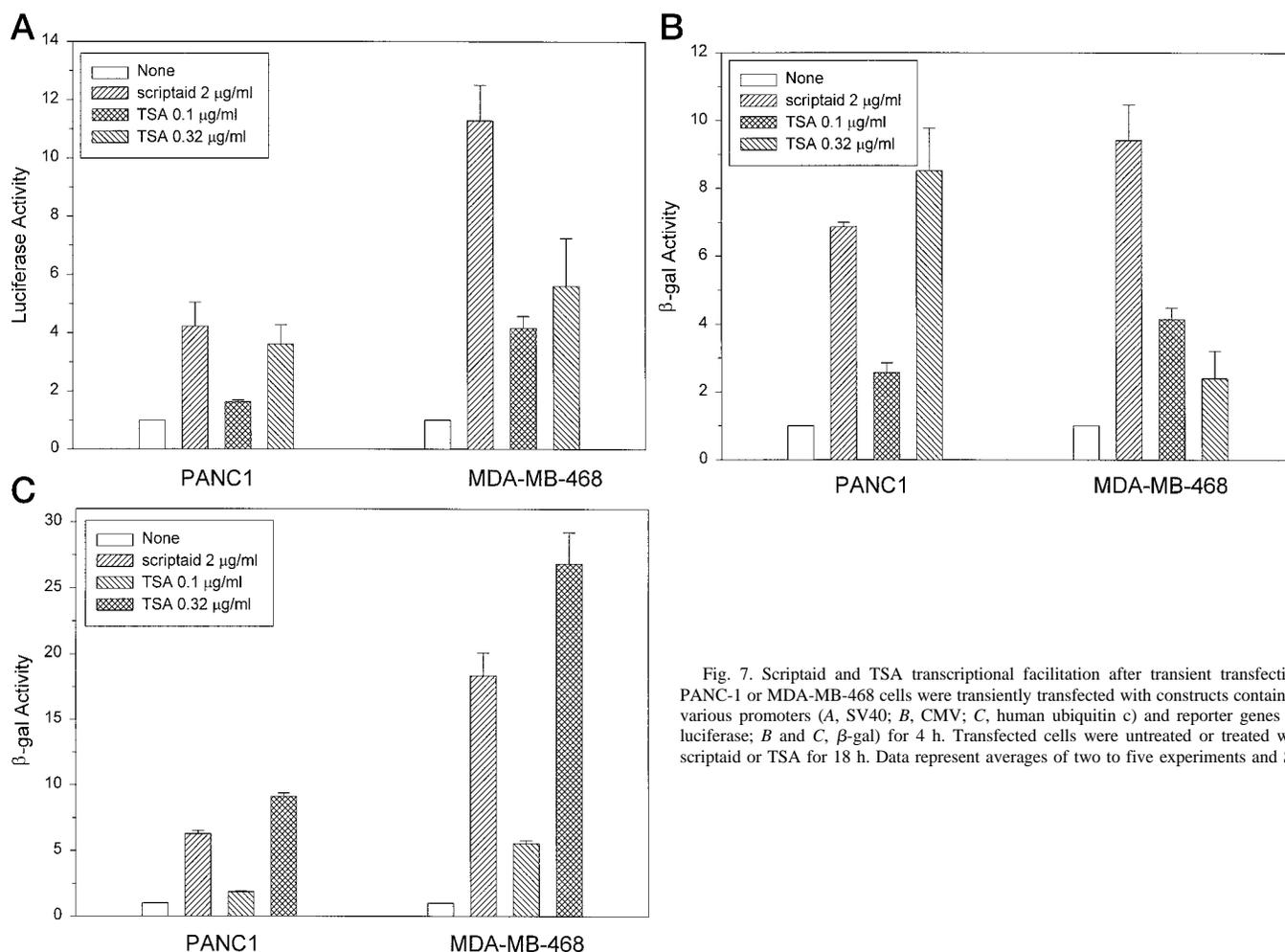


Fig. 7. Scriptaid and TSA transcriptional facilitation after transient transfection. PANC-1 or MDA-MB-468 cells were transiently transfected with constructs containing various promoters (A, SV40; B, CMV; C, human ubiquitin c) and reporter genes (A, luciferase; B and C, β -gal) for 4 h. Transfected cells were untreated or treated with scriptaid or TSA for 18 h. Data represent averages of two to five experiments and SE.

induction seen in an experimental situation may not always represent a 100% increase in strength of transactivation *per se* but could be mimicked, for example, by a 10% decrease in repression. These two possibilities could presumably be distinguished by the use of scriptaid and other HDAC inhibitors. In some reporter systems, negative effects on transcription (repression) may completely overshadow the positive effects. The use of HDAC inhibition to chemically dissect a pathway should unmask some important measures of pathway activation that could be overlooked in an undissected system. Indeed, it has been previously observed that the presence of TSA or butyrate indeed uncovered the inducibility of certain reporters that initially had appeared inactive (13, 14).

TSA and butyrate are the most well studied of the HDAC inhibitors for their effects on reporters or integrated genes. Yet, the potential of such general applications of HDAC inhibitors are somewhat controversial, perhaps because the properties of TSA and butyrate in the published reports had been confusing. Various limitations of TSA and butyrate in the applicability to transcriptional assays have been noted in endogenous genes and on the introduction of exogenous sequences. Butyrate and phenylbutyrate have many functions other than inhibiting HDACs; they have been reported to affect the posttranscriptional modification of other genes (15) and the depletion of glutamine (16). There are variable observations that conclude that TSA and other inhibitors do not consistently activate all of the promoters, and such failures of transcriptional facilitation have included the common general-utility promoters CMV and SV40 (17–19). Some of the reported transcriptional actions required a specific small recognition element

(20–22), or the activity of a particular coactivator (23). Furthermore, TSA is not always found to facilitate the detection of positive signal transduction events without interfering with the magnitude of relative transactivation activity (13, 22). It was, therefore, of interest that a more general utility could here be indicated, at least for some HDAC inhibitors within a defined system.

In summary, the identification of scriptaid confirmed the feasibility of compound screening in mammalian cells using this reporter system, in the absence of a formal compound-design effort. Scriptaid is shown to be a novel HDAC inhibitor with robust activity and relatively low toxicity, which suggests a wider utility in transactivation assays and in studies of histone acetylation.

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