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Ecteinascidin-743 Inhibits Activated but not Constitutive Transcription

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Abstract

Ecteinascidin-743 (ET-743) is a promising chemotherapeutic agent currently in Phase III clinical trials. Previous studies indicated a novel spectrum of activity for this agent, including transcriptional inhibition. Initially hypothesized to target a single transcription factor (NF-Y), we now show that ET-743 is a more general inhibitor of activated transcription. Induction of the Sp1-regulated *p21* gene by Trichostatin A (TSA) was blocked by ET-743 at concentrations that had minimal effect on uninduced (constitutive) expression. Moreover, ET-743 blocked induction of Gal4 fusion proteins by TSA without affecting activation mediated by the fusion proteins in the absence of the inducer. Finally, microarray analysis of SW620 cells treated with TSA and/or ET-743 indicated that activation of TSA-responsive promoters was blocked by ET-743 with little affect on nonresponsive promoters. These results, taken together with previous reports, leads us to suggest a mechanism whereby ET-743 is a novel, potent, and general inhibitor of activated but not uninduced transcription.

Introduction

ET-743,[4] a tetrahydroisoquinolone alkaloid derived from the marine tunicate *Ecteinascidia turbinata*, is a highly promising and potent antitumor agent. ET-743 is active against a variety of tumor cell lines growing in culture at subpicomolar to nanomolar concentrations, and exhibits remarkable antitumor activity in *in vivo* models (1–3). Several recent Phase I and II trials demonstrate that ET-743 has activity in a variety of soft tissue sarcomas when given to heavily pretreated patients (4). These preliminary data are encouraging, and Phase III trials of ET-743 as a single agent, as well as Phase I trials of ET-743 in combination with other agents, are currently underway in the United States and Europe.

The mechanism by which ET-743 exerts its cytotoxic effects has not yet been elucidated, but studies in several laboratories suggest a novel spectrum of activities. First, ET-743 binds to the minor groove of DNA and alkylates the N2 position of guanine (5, 6). This reaction appears to be DNA-sequence specific, with ET-743 preferring GC-rich triplets (5, 6); recent studies suggest that it is the off-rate, rather than the on-rate, which is influenced by DNA sequence (7). Whereas this is not uncommon for DNA binding agents, what sets ET-743 apart is that this interaction results in a bending of the DNA toward the major groove, rather than the minor groove as seen with other minor groove-interacting drugs (8). Second, ET-743 has been shown to be less toxic to cells that are defective in NER (9–12). This is in contrast to what is observed with most DNA damaging agents in which cells develop resistance through an enhancement of their NER mechanisms (13). That this has been shown to be peculiar to transcription-coupled NER rather than global NER (10) is intriguing in light of the third characteristic of ET-743, its ability to block transcriptional activation (14–16).

For the past few years, our laboratory has been investigating the role of ET-743 as a transcriptional inhibitor. These studies were prompted by an early analysis of the effect of ET-743 on the binding of minor groove proteins *in vitro*, where it was found that ET-743 affected the interaction of the trimeric transcription factor NF-Y with its cognate DNA element. We had shown previously that rapid induction of *MDR1* transcription by multiple inducers, including HDAC inhibitors (17), UV irradiation (18), and the MDR drug doxorubicin,[5] is mediated through an enhancer element, which interacts with NF-Y and the GC element binding proteins, Sp1 and Sp3. We refer to the protein complex that interacts at this element as the MDR1 enhancesome. NF-Y, apparently in cooperation with Sp1, recruits P/CAF,[6] a factor involved in chromatin remodeling, which in turn mediates a transcriptional response through its ability to acetylate histones and possibly NF-Y itself (19). Therefore, NF-Y is a central mediator of *MDR1* activation and likely functions, at least in part, by facilitating changes in chromatin structure in response to a variety of inducers.

The identification of NF-Y as an integral component in MDR1 activation prompted us to evaluate the effect of ET-743 on NF-Y-mediated activation of the *MDR1* promoter. We found that ET-743 blocked activation of *MDR1* by all of the inducers that converged on the *MDR1* enhancesome, with little affect on uninduced transcription, leading us to suggest that NF-Y may be a direct target of ET-743 action. This hypothesis was reinforced by a concurrent study by Minuzzo *et al.* (15) showing that heat shock activation of the NF-Y-regulated *hsp 70* promoter is also blocked by ET-743. However, several lines of evidence from our laboratory (14) and others (16) suggested that transcriptional inhibition by ET-743 was more complex than the inhibition of NF-Y binding that had been suggested by previous *in vitro* studies (20), and that NF-Y may not be the direct or sole target of ET-743 action. This has led us to readdress the mechanism by which ET-743 blocks transcription.

In the present study, we show that the effects of ET-743 are not limited to NF-Y-mediated transcription nor to the *MDR1* promoter. Indeed, activation of the p21 promoter, which is regulated by the major-groove binding protein Sp1 and is independent of NF-Y, is also inhibited by ET-743. Moreover, microarray analyses, as well as examination of transcriptional regulation by several Gal4-activator fusion proteins, suggest that ET-743 may be the prototype for a new class of agents that are inhibitors of activated but not constitutive transcription.

Materials and Methods

Cell Culture and Reagents. The human colon carcinoma cell line SW620 was maintained in RPMI 1640 supplemented with 10% (v/v) fetal bovine

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[4] The abbreviations used are: ET-743, Ecteinascidin-743; NER, nucleotide excision repair; HDAC, histone deacetylase; MDR, multidrug-resistance; TSA, trichostatin A.

[5] Z. Hu and K. W. Scotto, unpublished observations.

[6] S. Jin and K. W. Scotto, unpublished observations.

serum, 2.0 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Trichostatin A was purchased from Wako Biochemicals (Osaka, Japan). ET-743 was obtained from PharmaMar SA (Madrid, Spain) and was used in these studies at 50 nM, a concentration that results in approximately 50–60% growth inhibition with >90% cell viability at 24 h. The human p21 promoter construct, WWP-Luc, was obtained from Dr. Bert Vogelstein, Johns Hopkins University, Baltimore, MD. The 2.4 Kb HindIII fragment was removed and subcloned into the HindIII site of pGL2Basic to generate pGL-p21. The Sp1–3 site of pGL-p21 was mutated to create p21mSp1–3 using the Quik-Change site-directed mutagenesis kit (Stratagene). The oligonucleotides used to create the mutation were as follows, with the mutated bases in lowercase:

mSp1–3up: 5′-GAGCGCGGGTCCCGgaTCCTTGAGGCG-3′

mSp1–3lo: 5′-CGCCTCAAGGAtcCGGGACCCGCGCTC-3′.

Assays for mRNA and Promoter Activities. Nuclease protection assays of p21 and glyceraldehyde-3-phosphate dehydrogenase mRNA levels were performed essentially as described (14). For the *p21* promoter analysis, SW620 cells were allowed to grow for 4–6 h in MEM-Zinc Option (Life Technologies, Inc.) with 10% fetal bovine serum and then transiently transfected with *p21* promoter/luciferase reporter constructs (1 μg/well), along with 1 μg of salmon sperm DNA as carrier, using the calcium phosphate precipitation method. After 16 h, cells were allowed to recover in complete growth medium for 6 h and then treated with 100 ng/ml TSA and/or 50 nM ET-743, and incubated for an additional 24 h before harvesting. Luciferase and protein assays were performed as described previously (17). Luciferase activity was normalized to protein concentration. For analysis of the effect of ET-743 on transcriptional activation by the Gal4 fusion proteins, 0.4 μg of each Gal4 expression plasmid (1 ng of Gal4-VP16; Ref. 21) was cotransfected separately with 0.4 μg reporter plasmid (cfos::Luc) into SW620 cells using Lipofectin (Life Technologies, Inc.). Cells were incubated for 16–24 h after transfection, then TSA and/or ET-743 were added and cells were incubated for an additional 24 h before harvesting. Luciferase activity was determined and normalized to protein content.

Gel Shift Analysis. Sp1 was synthesized from pcDNA3/Sp1 using the TNT Quick Coupled Transcription/Translation kit (Promega). ET-743 and Sp1 were incubated in 30 mM HEPES (pH 7.9), 20% glycerol, 70 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 1% DMSO, and 2% polyethylene glycol 8000 with a [γ-^{32}P]ATP-labeled oligonucleotide containing the consensus Sp1 binding site. Complexes were resolved on a 4% nondenaturing polyacrylamide gel.

Microarray Analysis. SW620 cells were treated with TSA (100 ng/ml), ET-743 (50 nM), or a combination of both drugs for 12 h then harvested for preparation of total RNA using Trizol (Life Technologies, Inc.) according to the manufacturer's directions. For each treatment, a minimum of 300 μg of total RNA was provided to Incyte Technologies (St. Louis, MO) for extraction of polyadenylated RNA and subsequent analysis using the UniGEM Human V 2.0 microarray.

Results

ET-743 Blocks Transcriptional Activation Independent of NF-Y. Our analyses of the *MDR1* promoter have shown that activation by TSA requires both the NF-Y and Sp1 binding sites located within the proximal promoter region (the *MDR1* enhancesome).[6] Our earlier studies on the effect of ET-743 on activation of *MDR1* through the proximal enhancesome, taken together with other observations in the field (14, 15, 20), led us to propose NF-Y as a possible target for ET-743 action. However, we did not rule out the possibility that Sp1 or other factors could also play a role in ET-743 transcriptional inhibition. To test this possibility, the effect of ET-743 on the $p21^{WAF1/Cip1}$ promoter was evaluated. This promoter was chosen because: (*a*) it is induced by many of the same agents that activate the *MDR1* enhancesome, including TSA (22), butyrate (23), and UV (24); and (*b*) NF-Y is not involved in either basal or inducible transcription of $p21^{WAF1/Cip1}$; instead, induction is mediated by adjacent Sp1 sites (Fig. 1*A*). Activation of *p21* by TSA is mediated specifically through two of these sites, labeled Sp1–3 and Sp1–4, with the upstream site having a greater effect on induction (22).

Nuclease protection assays were performed on total RNA derived from SW620 cells treated for 24 h with either TSA or sodium butyrate with or without ET-743. Treatment with TSA or sodium butyrate induced expression of *p21* mRNA, similar to the effect seen on *MDR1*; this activation was blocked by concomitant treatment with ET-743 (Fig. 1*B*). To determine whether this effect occurred at the level of transcription, a full-length $p21^{WAF1/Cip1}$ promoter construct was transfected into SW620 cells (Fig. 1*C*). As reported previously, TSA activated this construct ~5-fold; this activation was abrogated by ET-743. Notably, ET-743 had little effect on uninduced activity of the promoter, reminiscent of what was observed with the *MDR1* promoter (14). Similar results were obtained using a *p21* promoter construct lacking sequences upstream of −215 (data not shown). Mutation of the Sp1–3 site in the context of the full-length promoter (p21mSp1–3) abolished activation by TSA; ET-743 had little effect on this construct, indicating that the Sp1 sites located in the proximal promoter region mediated both TSA activation and ET-743 inhibition. Thus, ET-743 inhibited p21 transcriptional activation independent of NF-Y, indicating that ET-743 can affect regulation mediated by transcriptional activators regardless of whether they interact with the minor groove (NF-Y) or major groove (Sp1) of DNA.

In light of this observation, we reevaluated the effect of ET-743 on the binding of Sp1 to its consensus sequence using a gel shift assay (Fig. 2). Increasing concentrations of ET-743 were added to an oligonucleotide containing the consensus Sp1 site either before the addition of *in vitro* translated Sp1 (Fig. 2*A*) or after incubation with Sp1 (Fig. 2*B*). Identification of Sp1 in the DNA-protein complex was accomplished in supershift assays using an Sp1-specific antibody (data not shown). ET-743 had no effect on Sp1 binding under either condition, even at concentrations above what is pharmacologically achievable, consistent with what has been reported previously (20). This indicates that it is unlikely that ET-743 blocks Sp1-mediated activation of the p21 promoter by interfering with transcription factor binding. To determine whether the effect of ET-743 on inhibition of activated transcription extended even beyond Sp1 and NF-Y, we took advantage of a transcription assay system which uses Gal4 fusion proteins in which the Gal4 DNA-binding domain is fused to the activation domain of a heterologous factor, and a reporter gene under the control of four Gal4-binding sites upstream of a minimal TATA-containing promoter. Five fusion proteins were tested: Gal4-VP16, Gal4-E2F1, Gal4-CTF, Gal4-Sp1, and Gal4-TBP (21). Four of these proteins represent two classes of transcription factors based on their specific role in the transcription process: type 1 proteins (Sp1 and CTF) stimulate initiation, whereas type IIB proteins (VP16 and E2F1) stimulate both initiation and elongation (25). The fifth protein, TBP, is required for basal transcription of all known promoter classes. The Gal4 fusion plasmids were cotransfected with the Gal4 reporter construct into SW620 cells, and the ability of each of the fusion proteins to activate transcription, in the presence and absence of ET-743, was examined. As shown in Fig. 3, all of the fusion proteins activated transcription compared with the Gal4 DNA-binding domain alone (Gal4). Treatment with TSA led to superactivation by two of the five fusion proteins relative to the Gal4-binding domain alone: Sp1 (~10-fold) and TBP (~20-fold). In both cases, ET-743 blocked this activation (Fig. 3). Most importantly, ET-743 had minimal effect on fusion protein-mediated transcription in the absence of TSA, adding additional support to the hypothesis that ET-743 specifically targets inducible transcription.

Our data thus far indicate that, rather than target a specific transcription factor as hypothesized previously, ET-743 may target a subset of factors that share a common mechanism of action or activation. To provide additional support for this new hypothesis, a microarray analysis was performed with RNA from cells treated with TSA alone, ET-743 alone, or TSA and ET-743, using the UniGEM Human V 2.0 gene chip (Incyte Technologies). As shown in Table 1,

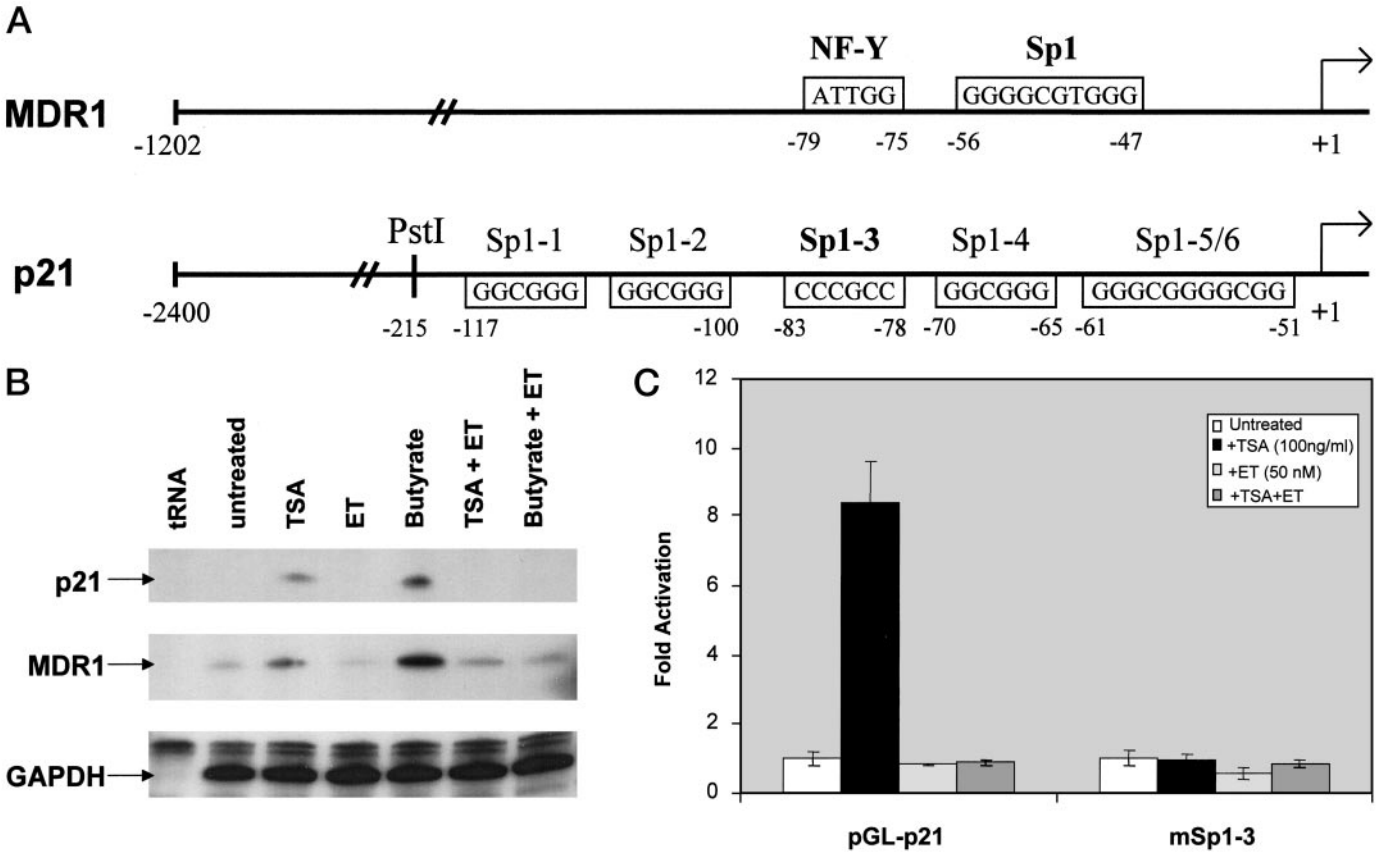

Fig. 1. *A*, sequence of the proximal *MDR1* and *p21* promoters. The NF-Y and Sp1 binding sites in the *MDR1* promoter, and the Sp1 binding sites in the *p21* promoter are *boxed*. The Sp1–3 site of the *p21* promoter, which mediates induction by TSA, is *bolded*. *B*, induction of *MDR1* and *p21 RNA* by TSA is inhibited by ET-743. Nuclease protection analysis of total RNA prepared from SW620 cells treated for 24 h with either TSA (100 ng/ml) or sodium butyrate (2 mM), and/or ET-743 (50 nM). *C*, induction of the *p21* promoter by TSA is inhibited by ET-743. A wild-type *p21* promoter reporter construct or a construct containing a mutation in the Sp1–3 site, were transiently transfected into SW620 cells. After a 24-h incubation with TSA and/or ET-743, luciferase activity was determined. Activity was normalized to protein content and is reported as fold activation over activity of untreated cells, which is set at 1. The data shown are representative of a minimum of two experiments performed in triplicate; *bars*, ±SD.

several genes were activated by TSA. Notably, activation of all of these genes was inhibited in the presence of ET-743, although again, the drug had minimal affect on uninduced transcription.

Discussion

Our previous studies on the effects of ET-743 on activation of the *MDR1* promoter by multiple inducers (TSA, sodium butyrate, and UV irradiation) had suggested that NF-Y, a critical mediator of this activation, could be a specific drug target (14). This was consistent with the observations that ET-743 preferentially abrogated the interaction of NF-Y with its binding site *in vitro* (20) and that NF-Y-regulated heat shock activation of the *hsp 70* gene was also inhibited by ET-743 (15). However, several observations at that time suggested that ET-743 did not affect the interaction of the transcription factor with the *MDR1* promoter and that NF-Y was not the direct, or only, transcriptional target of this novel agent: (*a*) the concentrations required for *in vivo* repression (nanomolar) were several orders of magnitude below what was required for *in vitro* inhibition of NF-Y binding (micromolar); (*b*) nuclear extracts prepared from ET-743-treated cells supported NF-Y complex formation indistinguishable from that observed in extracts from untreated cells; and (*c*) *MDR1* promoter-localized hyperacetylation, which is dependent on the recruitment of the histone acetylase P/CAF by promoter-bound NF-Y, was not affected by ET-743 (14). In light of this and as part of our ongoing effort to define the mechanism of transcriptional inhibition by ET-743, we have broadened our analyses to include NF-Y-independent, TSA-responsive promoters.

We now report that ET-743 can inhibit transcriptional activation mediated by Sp1, which shares with NF-Y the ability to mediate activation in response to HDAC inhibition but interacts with the major rather than the minor groove of DNA. Moreover, ET-743 inhibited activated transcription mediated by both Gal4-Sp1 and Gal4-TBP.

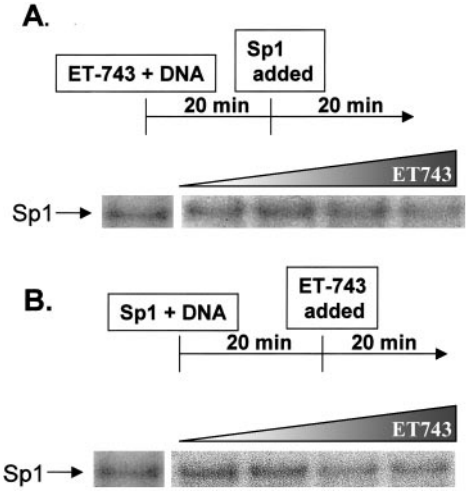

Fig. 2. ET-743 does not block Sp1-DNA interactions. Increasing concentrations of ET-743 (0.5 μM, 2 μM, 10 μM, and 40 μM) were added to a ^{32}P-labeled oligonucleotide containing the Sp1 binding site either (*A*) before or (*B*) after the addition of *in vitro*-translated Sp1.

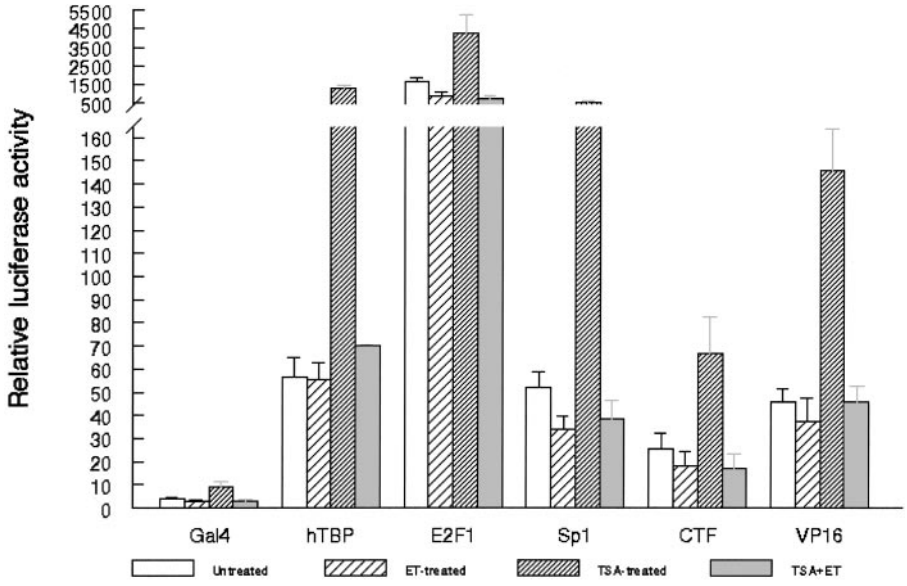

Fig. 3. ET-743 blocks activation of Gal4 fusion proteins by TSA without affecting uninduced transcription. Gal4 fusion proteins (*TBP*, *E2F1*, *Sp1*, *CTF*, and *VP16*) or a construct encoding the Gal4 binding domain alone (*Gal4*) were cotransfected into SW620 cells along with a luciferase reporter gene containing four Gal4 binding sites. Transfected cells were treated with TSA (100 ng/ml) and/or ET-743 (50 nM) 24 h before harvesting. Luciferase activity was determined, normalized to protein content, and is reported as relative luciferase units. The data shown are representative of a minimum of two experiments performed in triplicate; *bars*, ±SD.

Finally, microarray analysis indicates that ET-743 inhibits activation of the subset of genes induced upon HDAC inhibition. These studies, together with previous observations by our laboratory and others, suggest the following: (*a*) ET-743, which interacts with the minor groove of DNA, can affect transcriptional activation mediated by proteins interacting with either the minor groove (NF-Y and TBP) or the major groove (Sp1 and SXR; Ref. 16) of DNA; (*b*) uninduced transcription is not markedly affected by ET-743; this was seen in both the *p21* promoter assays and the Gal4 fusion studies, and is consistent with what we had observed previously with the *MDR1* promoter; (*c*) ET-743 does not appear to inhibit transcription by blocking the interaction of transcription factors with their DNA-binding elements, because activation of the Gal4 reporter gene by Gal4 fusion proteins in the absence of TSA induction was not affected by ET-743, indicating that the fusion protein remained bound and active in the presence of the drug. This latter observation makes it unlikely that disruption of direct transcription factor-DNA interactions is a mechanism of action of this drug.

The observation that activation mediated by a variety of DNA-binding proteins can be inhibited by ET-743, taken together with the data suggesting that ET-743 can block activation by seemingly disparate inducers (heat shock, radiation, chemotherapeutics, and HDAC inhibitors), suggest that ET-743 is a broader spectrum inhibitor of transcription than initially proposed. However, unlike other transcriptional inhibitors such as actinomycin D, ET-743 appears to be unique in its ability to inhibit activated transcription with minimal affect on uninduced transcription. How this is achieved is unclear. Our observation that activation of TSA-responsive genes can be inhibited by ET-743 suggests a chromatin-based model. Indeed, NF-Y (17, 19), TBP (26, 27), and Sp1 (28, 29) have all been shown to be part of multiprotein complexes that include histone-modifying enzymes and chromatin-remodeling proteins; it is likely that SXR, like other nuclear receptors (30, 31), is also a component of similar multiprotein complexes. Whether all of the chromatin-regulated genes can be targeted by ET-743 or whether a subset of these genes, perhaps those for which the critical promoter regions include the preferred binding motifs PuGG or PyGG (7), are susceptible to inhibition by this agent remains to be determined. Finally, the question as to whether components of the chromatin regulatory network are targets of this novel chemotherapeutic agent and how these targets may be linked to transcription-coupled NER awaits additional investigation.

Table 1 *Results of microarray analysis of SW620 cells treated with TSA alone ET-743 alone, or a combination of TSA and ET-743*

Changes in expression (+ or −) were determined relative to untreated cells. Only those genes with TSA-induced differences ≥2 are included in the table. Values in the range of −1.4 to +1.4 were not considered statistically different from 1.0.

TSA	ET	TSA/ET	RNA	Accession no.
8.7	−1.2	1.7	Short-chain alcohol dehydrogenase family member	NM005794
4.4	1	1.4	Metallothionein 1L	BF676799
4.2	1.1	1.4	RNA helicase-related protein	AF078844
3.2	1.4	1.9	Spermidine/spermine N1-acetyltransferase	AL047358
2.8	−1.2	−1.1	Protease, cysteine 1 (legumain)	NM005606
2.6	−1.2	1.6	Nuclear RNA export factor 1	U80073
2.3	1	−1.3	Upregulated by 1,25-dihydroxyvitamin D	AU137341
2.2	−1	1.1	Thymidine kinase, soluble	BE271054
2.2	−1.6	1.1	ADP-ribosylation factor-like 6 interacting protein	AU120805
2.2	−1.7	−1.5	Human glucose transporter pseudogene	M55536
2.1	1.1	1.5	Ornithine aminotransferase (gyrate atrophy)	M23204
2	−1.1	1	Silver (mouse homologue) like	BE892678
2	−1.1	1	Guanine nucleotide binding protein (G protein)	AL040479
2	1.1	1.1	Glucose-6-phosphate dehydrogenase	M12996

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