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Advances in Brief

Inhibition of Histone Deacetylase Activity by Trichostatin A Modulates Gene Expression during Mouse Embryogenesis without Apparent Toxicity[1]

Clara Nervi,[2] Ugo Borello, Francesco Fazi, Viviana Buffa, Pier Giuseppe Pelicci, and Giulio Cossu[2]

Department of Histology and Medical Embryology, University of Rome, 00161 Rome [C. N., U. B., F. F., V. B., G. C.]; Stem Cell Research Institute, Dibit, 20132 Milan [U. B., G. C.]; and European Institute of Oncology, Department of Experimental Oncology, 20141 Milan [P. G. P.], Italy

Abstract

Remodeling of the chromatin template by inhibition of histone deacetylase (HDAC) activities represents a major goal for transcriptional therapy in neoplastic diseases. Recently, a number of specific and potent HDAC-inhibitors that modulate *in vitro* cell growth and differentiation have been developed. In this study we analyzed the effect of trichostatin A (TSA), a specific and potent HDAC-inhibitor, on mouse embryos developing *in vivo*. When administered i.p. to pregnant mice (at a concentration of 0.5–1 mg/kg) at postimplantation stages (embryonic day 8 to embryonic day 10), TSA was not toxic for the mother and did not cause any obvious malformation during somitogenesis or at later stages of development. Treated embryos were born at similar frequency and were indistinguishable from control animals, developed normally, and were fertile. Interestingly, embryos from TSA-treated mice killed during somitogenesis were modestly but consistently larger than control embryos and presented an increased (+2 to +6) number of somites. This correlated with an increased acetylation of histone H4, the number of somites expressing the myogenic factor *Myf-5*, and the expression of *Notch*, *RARα2*, and *RARβ2* mRNAs. These data indicate that the effects of TSA on transcription: (*a*) are not toxic for the mother; (*b*) transiently accelerated growth in mouse embryos without perturbing embryogenesis; and (*c*) do not result in teratogenesis, at least in rodents. Thus, TSA might represent a nontoxic and effective agent for the transcriptional therapy of neoplasia.

Introduction

A common multiprotein complex containing catalytically active HDACs[3] mediates the transcriptional repression of transcription factors such as Mad/MAX, E2F, YY1, or members of the steroid/thyroid receptor superfamilies (1–3). Deacetylation of nucleosomal core histone tails by the HDACs leads to a chromatin conformation that inhibits transcription, whereas histone acetylation results in transcriptional activation after local chromatin remodeling and recruitment of the RNA polymerase II complexes (2, 3).

Drugs modulating the acetylation status of histones have been proposed recently as a novel approach for the molecular treatment of cancer (4, 5). In AMLs, different chromosomal translocation-generated fusion gene products, such as PML/RARα, PLZF/RARα, and AML1/ETO, induce an abnormal regulation of HDAC activities, an event that plays a crucial role in the pathogenesis and maintenance of the transformed phenotype (6–10). In agreement with these biological findings, clinical efficacy has been reported for the HDAC-inhibitor butyrate and its analogue phenylbutyrate in the treatment of AML and in a RA-resistant acute promyelocytic leukemia patient (11, 12). A potential use of HDAC-inhibitors is also foreseen for cancer, with inactivation of tumor suppressor genes attributable to the hypermethylation of their promoters. HDAC, indeed, is involved in the transcriptional silencing of methylated promoters (3, 13).

However, the availability of HDAC-inhibitors for clinical use is limited. Butyrates and their derivatives have limited toxicity, but they act as nonspecific, transient, and reversible HDAC-inhibitors. Recently, a number of highly specific, stable, and more potent inhibitors of HDAC activities have been developed (14). Among these, TSA is one of the most active and studied. TSA has been found effective in modulating cell growth and differentiation in a number of *in vitro* systems, including carcinoma cell lines and myeloid leukemias, suggesting its potential use in transcriptional therapy of neoplasia (4, 6, 15–17).

A major issue concerning transcriptional therapy with HDAC-inhibitors is the expected toxicity of these compounds. Indeed, the general involvement of HDACs in a variety of fundamental cellular processes suggests a wide range of biological effects for HDAC-inhibitors when used as drugs. In support of this view, a recent publication reported malformations in embryos treated with TSA *in vitro* (18). However, a systematic analysis of the toxicity of HDAC-inhibitors during embryo development and in the adult life is lacking. In this study, we investigated the effect of TSA treatment in adult mice and during embryonic development and report that, despite its wide effects on histone acetylation and gene expression, TSA is not toxic and does not perturb mouse embryonic or postnatal development.

Materials and Methods

CD1 pregnant mice (E 8, 9, and 10) received i.p. injections of 15 μg of TSA (WAKO Chemicals, Neuss, Germany) in 0.2 ml of DMSO (Sigma, Milano, Italy) or 0.2 ml of vehicle alone. In some experiments, the same dose was repeated after 24 h. At the time indicated, mice were killed, and the embryos were observed under a dissecting stereomicroscope to evaluate their viability, the presence of external malformations, and the number of somites. Embryos were then fixed in 4% paraformaldehyde for histology, X-Gal staining, and whole-mount *in situ* hybridization using Notch and RARα1, -α 2, and -β 2 antisense probes (19–22). Immunoblot analysis was performed on total embryo homogenates (60 μg) using anti-histone H4 and anti-acetylated histone H4 antibodies (Upstate Biotecnology). Densitrometic analysis was performed using the Advanced Image Data Analyzer (Raytest, Milan, Italy).

Results and Discussion

The administration of a single TSA i.p. injection (15 μg) to pregnant CD1 outbred mice at E 8, 9, or 10 did not result in any apparent toxicity for the mother and did not cause embryo malformations or defects at birth or during the animals' postnatal growth. Animals treated *in utero* developed to normal adult size and were fertile.

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[2] To whom requests for reprints should be addressed, at Department of Histology and Medical Embryology, University of Rome, "La Sapienza," 00161 Rome, Italy. Tel: 39-06-497681026757; Fax: 39-06-4462854; E-mail: clara.nervi@uniroma1.it or giulio.cossu@uniroma1.it.

[3] The abbreviations used are: HDAC, histone deacetylase; AML, acute myeloid leukemia; TSA, trichostatin A; RA, retinoic acid; RAR, RA receptor; RARE, RA response element; E, embryonic day; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

Observation under a dissecting microscope revealed that 6- to 24-h TSA treatment of the mother constantly resulted in a slight, but statistically significant, increase in the number of somites in E 8.5-, 9-, or 9.5-treated embryos. Treatment with the vehicle (DMSO) induced no variations in any of the parameters analyzed (control; Fig. 1*A*). From E 8 to E 11, the number of somites represents the simplest and most faithful indicator of the embryonic stage of development.

We investigated whether the effect of TSA on embryos correlated with modifications of histone acetylation. Fig. 1*B* shows an immunoblotting analysis of extracts prepared from three control and three TSA-treated embryos using an antibody that recognizes the acetylated form of histone H4. Although these forms were already detectable in control whole embryos, they appeared more abundant in embryos from TSA-treated mice; this did not reflect an increased accumulation of the H4 protein as shown by an antibody recognizing the histone H4 protein in the same samples (Fig. 1, *B* and *C*). It appeared therefore that *in vivo* treatment with TSA affects mammalian embryonic development but is not embryotoxic. This is at variance with a previous study (18) that showed malformations in early postimplantation embryos exposed *in vitro* to TSA. In this case, direct exposure to high TSA concentrations of embryos, which in culture develop under critical conditions, may have caused effects that are not seen after exposure *in vivo*. Indeed, even repeating TSA administration after 24 h did not result in apparent abnormalities in treated embryos. This discrepancy might be attributable to the actual concentrations of TSA, which may not be directly comparable between the two studies.

During vertebrate embryogenesis, somites are formed in a strict cranio-caudal order by the successive segmentation of the paraxial mesoderm flanking the neural tube (23). Caudal to the most recently formed somite, the paraxial mesoderm appears morphologically unsegmented. Subsequently, somites become compartmentalized into a dorsal epithelial dermomyotome (future muscle and dermis) and a ventral mesenchymal sclerotome (future vertebral column and ribs). We therefore evaluated whether i.p. injection of TSA could modulate *in vivo* the expression of *Myf-5* and *Notch*, two genes involved in somitogenesis (24). *Myf-5* is a basic-helix-loop-helix transcriptional regulator of muscle determination and is a useful marker of somites that contain cells already committed to skeletal myogenesis (25). Once formed, the somites follow a normal process of maturation, and *Myf-5* is activated at the proper time, *i.e.*, in the second- to third-last-formed somite. *Myf-5* expression was evaluated by staining for X-Gal Myf-5/nlacZ (Myf-$5^{a2+/-}$) embryos, where the *nLacZ* reporter gene has been targeted into the *Myf*-5 locus (26). Fig. 2 (*A* and *B*) shows that both the total number of somites and the number of Myf-5-expressing somites (revealed in blue by X-Gal staining in *A*) were consistently increased in TSA-treated embryos.

By whole-mount *in situ* hybridization we found that Notch expression was increased in the presomitic paraxial mesoderm of TSA-treated embryos (revealed in purple by the antisense probe; Fig. 2*A*; a higher magnification of the presomitic mesoderm is shown in *C*). Because Notch and members of its signal transduction pathway con-

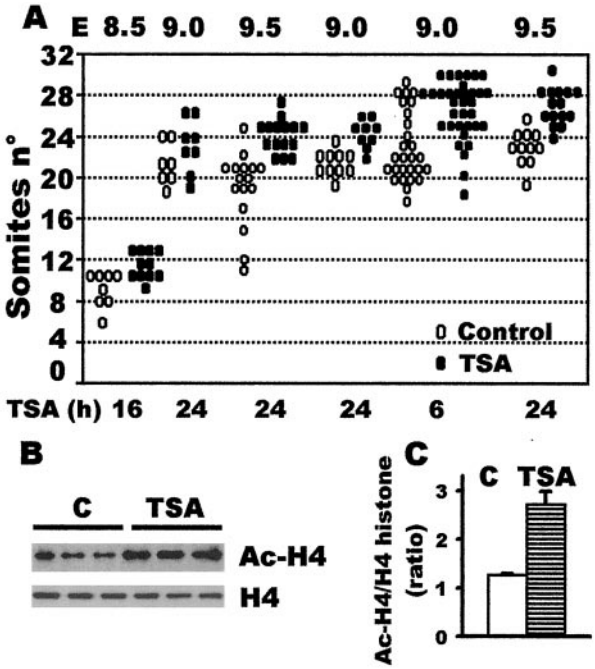

Fig. 1. *A*, number of somites in control (*open symbols*) and TSA-treated (*closed symbols*) embryos. The number of somites was counted, including the two occipital somitomers. Time of killing is indicated in the *upper* abscissa, and the length of previous TSA treatment is indicated in the *lower* abscissa. *B*, immunoblot analysis of the acetylation status of histone H4 was performed in total embryo extracts using the antiacetylated histone H4 antibody. Representative samples from three controls and TSA-treated embryos are shown. Expression of histone H4 was used to control the histone H4 acetylation levels. *C*, ratio Ac-H4:H4 histone in control (*open bars*) and in TSA-treated embryos (*dashed bars*).

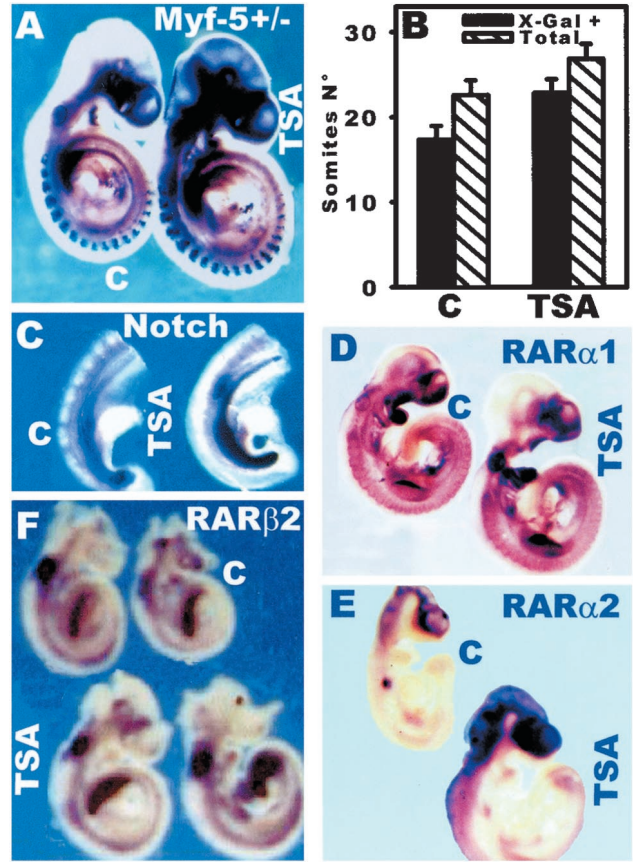

Fig. 2. Gene expression in TSA-treated embryos. Note than in all panels, TSA-treated embryos are slightly larger than control embryos. *A*, whole mount *in situ* hybridization for Notch on a control and a TSA-treated embryo, also stained for Myf-5 expression by X-Gal (28). Note that the TSA-treated embryo has two additional somites expressing Myf-5. This is quantified in *B*, where the total number of somites (*dashed bars*) and the number of X-Gal somites expressing Myf-5 (*closed bars*) are shown. Somites were scored as Myf5+ when they contained in excess of five X-Gal+ nuclei. Treated embryos had a higher level of Notch expression in the presomitic mesoderm; this is more evident in *C*, where only the dissected caudal portion of embryos hybridized with the Notch probe are shown. *D*, whole-mount *in situ* hybridization for RARα1 in control and TSA-treated E-9.5 embryos. Note comparable levels of expression throughout the mesoderm and in the branchial arches. *E*, whole-mount *in situ* hybridization for RARα2 in control and TSA-treated E-9.5 embryos. TSA-treated embryos expressed higher levels of RARα2 in the cranial spinal cord and in the hindbrain. *F*, whole-mount *in situ* hybridization for RARβ2 in two controls (*above*) and in two TSA-treated (*below*) E-9.5 embryos. Note that TSA-treated embryos express higher levels of RARβ in the presomitic mesoderm. *C*, control.

trol numerous cell-fate decisions during development and are involved in the patterning of somites (24), its increased expression might accelerate this process, resulting in the additional somites formed in response to TSA treatment. Together these results show that TSA treatment during the postimplantation stage, when critical morphogenetic events occur, results in the increased expression of Notch, accelerated formation of somites, and normal commitment to myogenesis.

Recently, a number of studies showed that unliganded nuclear receptors, such as the RARs (RARα, -β, and -γ), recruit the nuclear corepressors N-CoR and SMRT that associate with adapter proteins like Sin3 and histone deacetylases (HDAC1 and HDAC2) to form a transcriptional repressor complex at the response element of RA target genes (RAREs). (1, 2). Ligand binding releases corepressors and HDACs and recruits transcriptional adapters and coactivators that include histone acetyltransferase activities such as p300/CBP, ACTR, and TIF2 (1, 2). Interestingly, cells derived from mice lacking a functional *p300* gene proliferate poorly and display specific transcriptional defects of RA signaling (27). Each *RAR* gene generates distinct mRNA isoforms by the use of different promoters and alternative splicing. The RAR isoforms RARα2 and RARβ2 posses a RARE in their promoters that is responsible for their RA inducibility occurring through a HDAC-dependent signaling pathway (1, 20, 21). The RARE is not present in the promoter of the *RARα1* isoform, which, at variance with *RARα2*, is expressed constitutively in mouse adult tissues as a housekeeping gene and is widely distributed during mouse embryogenesis (20–22). We therefore examined by whole-mount *in situ* hybridization the expression levels of RARα1, RARα2, and RARβ2 in control and in TSA-treated E-9.5 mouse embryos. Fig. 2*D* shows similar levels of RARα1 transcript in the expected sites of expression, such as the branchial arches, somitic mesoderm, and limb buds of embryos from control and TSA-treated mice. In contrast, significantly higher levels of expression of RARα2 in the spinal cord and hindbrain, as well as of RAR-β2 in the presomitic mesoderm, could be detected in TSA-treated embryos when compared with control embryos. Thus, the HDAC-inhibitor TSA relieved a repressive activity associated with the HDAC-complex at the RARE of RA target genes.

In summary, the results presented in this study indicate that *in vivo* modulation of gene transcription by inhibition of HDAC activity by TSA treatment is neither toxic nor teratogenic, at least in rodents, suggesting that TSA might represent a novel and safe transcriptional therapeutical agent.

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References

1. Chambon, P. A decade of molecular biology of retinoic acid receptors. FASEB J., *10:* 940–954, 1996.
2. Glass, C. K., and Rosenfeld, M. G. The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev., *14:* 121–141, 2000.
3. Kouzarides, T. Histone acetylases and deacetylases in cell proliferation. Curr. Opin. Genet. Dev., *9:* 40–48, 1999.
4. Kosugi, H., Towatari, M., Hatano, S., Kitamura, K., Kiyoi, H., Kinoshita, T., Tanimoto, M., Murate, T., Kawashima, K., Saito, H., and Naoe, T. Histone deacetylase inhibitors are the potent inducer/enhancer of differentiation in acute myeloid leukemia: a new approach to anti-leukemia therapy. Leukemia, *13:* 1316–1324, 1999.
5. Redner, R. L., Wang, J., and Liu, J. M. Chromatin remodeling and leukemia: new therapeutic paradigms. Blood, *94:* 417–428, 1999.
6. Grignani, F., De Matteis, S., Nervi, C., Tomassoni, L., Gelmetti, V., Cioce, M., Fanelli, M., Ruthardt, M., Ferrara, F. F., Zamir, I., Seiser, C., Grignani, Fa., Lazar, M. A., Minucci, S., and Pelicci, P. G. Fusion proteins of the retinoic acid receptor-α recruit histone deacetylase in promyelocytic leukaemia. Nature (Lond.), *391:* 815–818, 1998.
7. Lin, R. J., Nagy, L., Inoue, S., Shao, W., Miller, W. H. J., and Evans, R. M. Role of the histone deacetylase complex in acute promyelocytic leukaemia. Nature (Lond.), *391:* 811–814, 1998.
8. Gelmetti, V., Zhang, J., Fanelli, M., Minucci, S., Pelicci, P. G., and Lazar, M. A. Aberrant recruitment of the nuclear receptor corepressor-histone deacetylase complex by the acute myeloid leukemia fusion partner ETO. Mol. Cell. Biol., *18:* 7185–7192, 1998.
9. Wang, J., Hoshino, T., Redner, R. L., Kajigaya, S., and Liu, J. M. ETO, fusion partner in t(8;21) acute myeloid leukemia, represses transcription by interaction with the human N-CoR/mSin3/HDAC1 complex. Proc. Natl. Acad. Sci. USA, *95:* 10860–10865, 1998.
10. Minucci, S., Maccarana, M., Cioce, M., De Luca, P., Gelmetti, V., Segalla, S., Di Croce, L., Giavara, S., Matteucci, C., Gobbi, A., Bianchini, A., Colombo, E., Schiavoni, I., Badaracco, G., Hu, X., Lazar, M. A., Landsberger, N., Nervi, C., and Pelicci, P. G. Oligomerization of RAR and AML1 transcription factors as a novel mechanism of oncogenic activation. Mol. Cell, *5:* 811–820, 2000.
11. Novogrodsky, A., Dvir, A., Ravid, A., Shkolnik, T., Stenzel, K. H., Rubin, A. L., and Zaizov, R. Effect of polar organic compounds on leukemic cells. Butyrate-induced partial remission of acute myelogenous leukemia in child. Cancer (Phila.), *51:* 9–14, 1983.
12. Warrell, R. P., Jr., He, L. Z., Richon, V., Calleja, E., and Pandolfi, P. P. Therapeutic targeting of transcription in acute promyelocytic leukemia by use of an inhibitor of histone deacetylase. J. Natl. Cancer Inst., *90:* 1621–1625, 1998.
13. Ng, H. H., and Bird, A. DNA methylation and chromatin modification. Curr. Op. Genet. Dev., *9:* 158–163, 1999.
14. Yoshida, M., Horinouchi, S., and Beppu, T. Trichostatin A and trapoxin: novel chemical probes for the role of histone acetylation in chromatin structure and function. Bioessays, *17:* 423–430, 1995.
15. Minucci, S., Horn, V., Bhattacharyya, N., Russanova, V., Ogryzko, V. V., Gabriele, L., Howard, B. H., and Ozato, K. A histone deacetylase inhibitor potentiates retinoid receptor action in embryonal carcinoma cells. Proc. Natl. Acad. Sci. USA, *94:* 11295–11300, 1997.
16. Saunders, N., Dicker, A., Popa, C., Jones, S., and Dahler, A. Histone deacetylase inhibitors as potential anti-skin cancer agents. Cancer Res., *59:* 399–404, 1999.
17. Ferrara, F. F., Fazi, F., Bianchini, A., Padula, F., Gelmetti, V., Minucci, S., Mancini, M., Pelicci, P. G., Lo Coco, F., Nervi, C. Histone deacetylase treatment restores retinoic acid signaling and differentiation in acute myeloid leukemia. Cancer Res., *61:* 2–7, 2001.
18. Svensson, K., Mattsson, R., James, T. C., Wentzel, P., Pilartz, M., MacLaughlin, J., Miller, S. J., Olsson, T., Eriksson, U. J., and Ohlsson, R. The paternal allele of the *H19* gene is progressively silenced during early mouse development: the acetylation status of histones may be involved in the generation of variegated expression patterns. Development, *125:* 61–69, 1998.
19. Reaume, A. G., Conlon, R. A, Zirngibl, R., Yamaguchi, T. P., and Rossant, J. Expression analysis of a Notch homologue in the mouse embryo. Dev. Biol., *154:* 377–387, 1992.
20. Leroy, P., Krust, A., Zelent, A., Mendelsohn, C., Garnier, J. M., Kastner, P., Dierich, A., and Chambon, P. Multiple isoforms of the mouse retinoic acid receptor α are generated by alternative splicing and differential induction by retinoic acid. EMBO J., *10:* 59–69, 1991.
21. Zelent, A., Mendelsohn, C., Kastner, P., Krust, A., Garnier, J. M., Ruffenach, F., Leroy, P., and Chambon, P. Differentially expressed isoforms of the mouse retinoic acid receptor β generated by usage of two promoters and alternative splicing. EMBO J., *10:* 71–81, 1991.
22. Mollard, R., Viville, S., Ward, S. J., Decimo, D., Chambon, P., and Dolle, P. Tissue-specific expression of retinoic acid receptor isoform transcripts in the mouse embryo. Mech. Dev., *94:* 223–232, 2000.
23. Keynes, R. J., and Stern, C. Mechanisms of vertebrate segmentation. Development, *103:* 413–429, 1988.
24. Pourquie, O. Segmentation of the paraxial mesoderm and vertebrate somitogenesis. Curr. Top. Dev. Biol., *47:* 81–105, 2000.
25. Tajbakhsh, S., and Cossu, G. Establishing myogenic identity during somitogenesis. Curr. Op. Genet. Dev., *7:* 634–641, 1997.
26. Tajbakhsh, S., Rocancourt, D., Cossu, G., and Buckingham, M. Redefining the genetic hierarchies controlling skeletal myogenesis: Pax-3 and Myf-5 act upstream of MyoD. Cell, *89:* 127–138, 1997.
27. Yao, T-P., Oh, S. P., Fuchs, M., Zhou, N-D., Ch'ng, L-E., Newsome, D., Bronson, R. T., Li, E., Livingston, D. M., and Eckner, R. Gene dosage-dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300. Cell *93:* 361–372, 1998.
28. Borello, U., Coletta, M., Tajbakhsh, S., Leyns, L., De Robertis, E. M., Buckingham, M., and Cossu, G. Transplacental delivery of the Wnt antagonist Frzb1 inhibits development of caudal paraxial mesoderm and skeletal myogenesis in mouse embryos. Development, *126:* 4247–4255, 1999.