The HDAC inhibitor depsipeptide transactivates the p53/p21 pathway by inducing DNA damage

Haiying Wang a,1, Wen Zhou a,1, Zhixing Zheng a, Ping Zhang a, Bo Tu a, Qihua He b, Wei-Guo Zhu a,∗

a Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Biochemistry and Molecular Biology, Peking University Health Science Center, 38 Xueyuan Road, Beijing 100191, China

b Department of Pathology and Biomedical Teaching Laboratory Center, Peking University Health Science Center, Beijing 100191, China

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1. Introduction

Post-translational modifications (PTMs) of histone tails are associated with changes in histone structure and thereby affect gene expression [1,2]. Histone acetylation, which directly relates to the activation of gene transcription, is the most extensively studied PTM [3,4]. Two classes of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs), determine the acetylation status of histones [5]. Increases in histone acetylation and decreases in histone deacetylation are well associated with the expression of tumor suppressor genes, which is thus beneficial for cancer treatment [6].

Because HDACs have been identified as molecular targets in cancer therapy [7], HDAC inhibitors (HDACi) are an effective therapeutic agent to kill cancer cells by either inhibiting HDAC activity or altering gene transcription to cause growth arrest, differentiation and/or apoptosis of tumor cells [8,9]. Histone deacetylases are classified into three classes according to their structure and cellular localization [10]. Depsipeptide, (also known as FK228 or FR901228) is a more potent inhibitor of class I HDACs than other HDAC inhibitors, such as trichostatin A (TSA) and sodium butyrate [11], and depsipeptide is a promising anticancer agent for both mono- and polytherapy [10,12]. In addition to inducing histone acetylation, an activity common to all HDAC inhibitors, depsipeptide shows other biological functions through varying mechanisms of action [10]. For example, depsipeptide induces acetylation of non-histone proteins, such as p53 and Hsp90 [13,14].

Depsipeptide also cooperates with nucleoside analogs, such as 5-aza-2′-deoxycytidine (5-aza-Cdr) and cytarabine (Ara-C), to activate transcription and to induce apoptosis. Apoptosis probably results from suppressing the repair of 5-aza-Cdr induced DNA damage [15]. In addition, we previously found that depsipeptide is a DNA demethylating agent [16], and this activity has prompted the exploration of depsipeptide in a much broader scientific context. However, the precise molecular mechanism of its anticancer effect remains obscure. Based on these factors, it is essential to...
understand the mechanism of the action of depsipeptide in human cancer cells.

Almost all HDAC inhibitors induce a significant increase in the expression of p21 [10,17,18], a protein that arrests the cancer cell cycle in the G1 phase. Interestingly, although p21 is a critical target of p53, HDAC inhibitor-induced p21 expression has been reported to be independent of p53 activation [19–21], however, we previously found that depsipeptide induces p21 expression by activating the p53 pathways [13]. Unlike other HDAC inhibitors, such as TSA, which induce p53 acetylation only by working with ionizing radiation or nicotinamide, an inhibitor of class III HDACs [22], depsipeptide alone, without cooperation from other agents, can induce acetylation of p53 at K373/382 and significantly increase p53 transactivity and p21 expression [13]. These differences between depsipeptide and other HDAC inhibitors in activating p53 or p21 expression lead us to further investigate the mechanism by which depsipeptide activates p53 activity.

As a transcription factor, depending on the nature of the stress, p53 can induce expression of many different downstream genes including p21, GADD45, and Bax to elicit various responses, such as cell cycle arrest, apoptosis, and DNA repair [23–25]. p53 exists in a latent conformation in unstressed cells because p53’s C-terminal tail allosterically interacts with its core DNA binding domain and negatively regulates its sequence-specific DNA binding transcription activation [26]. Upon stress, the interaction and negative regulation is abolished by p53’s posttranslational modifications, such as phosphorylation and acetylation [27]. The preeminent postmodifications is the direct phosphorylation of p53. Phosphorylations at the p53C terminus such as Ser315 and Ser392 are reported to regulate the oligomerization state and sequence-specific DNA binding ability of p53 [28,29]. In addition, phosphorylation and acetylation modification of p53 are interrelated [30]. Phosphorylation of p53 is important for efficient binding with CBP/p300 and PCAF complexes. For example, phosphorylation at N-terminal serines, such as Ser15, Ser33, and Ser37 has been reported to recruit p300/CBP and PCAF to induce p53 acetylation in response to DNA damage [31,32]. Phosphorylation of p53 at Ser20 or Thr18 can stabilize the p300–p53 complex and induce p53 acetylation [33]. Recently, it was reported that p53 C-terminal phosphorylation also modulates C-terminal acetylation in response to DNA damage [34]. Our study also found that p53 phosphorylation at Ser15 in turn results in acetylation of p53 at Lys320 and Lys373/Lys382 through p300 and CBP respectively, which are directly responsible for 5-aza-Cdr induced p21 expression [35].

In the present study, we conducted experiments to test how depsipeptide activates p53 transactivity and demonstrated that depsipeptide induces DNA damage by enhancing the production of reactive oxygen species (ROS), an effect that results from inhibiting the expression of thioredoxin reductase (TrxR). Subsequently, the accumulated ROS induces DNA damage, which in turn induces p53 phosphorylation at Thr18 and p53 acetylation at K373/382 as well as an increase in p21 expression.

2. Materials and methods

2.1. Cell culture and chemical treatments

Human lung cancer cell lines A549 and H1299 and the human colon cancer cell line HCT116 were grown in RPMI 1640 supplemented with 10% fetal bovine serum (heat inactivated at 56 °C for 45 min) and the appropriate amount of penicillin/streptomycin in a 37 °C incubator with a humidified, 5% CO2 atmosphere. The HDAC inhibitor depsipeptide, at 0.05 or 0.1 μM, was added to these cells for 0 to 12 h, and cells were then harvested for experiments.

2.2. Western blotting

Protein expression was detected by Western blotting as previously described. Cells were lysed with lysis buffer [50 mM Tris–HCl, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1% Igepal CA-630, and a mixture of protease inhibitors (Roche Diagnostics, Mannheim, Germany)]. Equal amounts of protein per sample (100–150 μg) were size-fractionated by 6–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The antibodies used were anti-p21 (F-5; Santa Cruz), anti-p53 (DO-1; Santa Cruz), anti-p300 (H-272; Santa Cruz), anti-β-actin (Santa Cruz), anti-acetyl-p53 (Lys373 and -382; Upstate), anti-phospho-p53 (Ser15, Ser20, Ser37, Ser392 and Thr18, Cell Signaling), anti-TrxR, anti-TrxR (Santa Cruz) and anti-H3 (Cell Signaling) antibodies.

2.3. Alkaline comet assay to detect DNA strand breaks

The alkaline comet assay, also known as the single-cell gel electrophoresis assay, was performed as described previously [36]. In brief, fully frosted microscope slides were covered with 110 μl of 0.5% normal melting agarose at 60 °C. The slides were immediately covered with cover slips and were then maintained at 4 °C for 15 min to allow the agarose to solidify. About 106 cells (depsipeptide treated or untreated) in 40 μl of PBS were mixed with an equal amount (40 μl) of 1% low melting agarose to form a cell suspension. After the cover slips were gently removed from the slides, the cell suspensions were pipetted onto the first agarose layer, spread and covered with cover slips, and maintained at 4 °C for 15 min to allow solidification. After removal of the cover slips, the slides were immersed in freshly prepared cold lysis solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris, pH 10.0. 1% sodium sarcosinate) with 1% Triton X-100 for 40 min at 4 °C. The slides were then placed in a horizontal gel electrophoresis tank filled with fresh electrophoresis solution (1 mM Na2EDTA, 300 mM NaOH, pH 13.0) for 10 min. The slides were then rinsed twice in Tris buffer (0.4 M Tris–HCl, pH 7.5) for 15 min (to neutralize the excess alkaline) at 4 °C. The slides were then stained with 75 μl of propidium iodide (5 μg/ml) for 30 min.

The slides were examined, and pictures were taken under a fluorescence microscope (TCS; Leica, Mannheim, Germany). To score the percentage of DNA in the comet tail, the CometScore automatic comet assay system was used (TriTek Corp, USA). The percentage of comet tail area (DNA tail area/total DNA area) and the comet tail length (from the center of the DNA head to the end of the DNA tail) were analyzed for 50 cells per slide.

2.4. ROS detection

Dihydroethidium (DHE), a redox sensitive fluorescent probe for superoxide, was used to detect ROS accumulation. After treatment, cells were incubated with 5 μM DHE (Sigma) for 30 min, after which they were washed, and ROS generation images were obtained by confocal laser microscopy and analyzed by an image analysis system (Q550CW; Leica).

2.5. Site-directed mutagenesis

Constructs for two p53 mutants (p53S37A and p53T18A) were generated using a site-directed mutagenesis kit (QuikChange; Stratagene, La Jolla, CA). A wild-type p53 expression vector (PClneo with full-length p53 cDNA) [37] was used as the mutagenesis template. The following primers were used for the mutagenesis: p53–S37A-up, 5′–CTG TCC CCC TTC CCG C(T)CC CAA GCA ATG GAT G–3′; p53–S37A-down, 5′–CAT CCA TTG CTT GGG C(A)C GCA AGG GGA ACA G–3′; p53–T18A-up, 5′–CCC TTC TGA GTC AGG AA(A) CAT TTG CAC ACC TAT G–3′; and p53–T18A-down, 5′–CAT AGG TCT
GAA AAT GCT[T]T TCC TGA CTC AGA GGG G-3'. In these primers, underlined italic nucleotides indicate the mutated nucleotides, and parentheses indicate the original nucleotides.

2.6. Chromatin immunoprecipitation (ChiP) assay

Cells from the A549 line were cross-linked with 1% formaldehyde for 10 min at 37 °C and then washed with cold PBS. The cell pellet was resuspended in 0.3 ml of lysis buffer (1% SDS, 100 mM NaCl, 50 mM Tris–HCl, pH 8.1, 5 mM EDTA), followed by sonication to an average DNA length of 500–1000 bp. Antibodies were added to each of the samples, which were then rotated at 4 °C overnight. After interaction with protein A beads and incubation overnight at 65 °C to reverse the cross-links, the DNA was dissolved in Tris-EDTA buffer and analyzed by PCR. The antibody anti-p53 (DO-1) was added separately to reaction solutions. Primers used for PCR were from the p21 promoter sequence: 5'-CTC ACA TCC TTC TTG AGA-3' (sense) and 5'-CAC ACA CAG AAT CTG ACT CCC-3' (antisense).

2.7. Co-immunoprecipitation assay (Co-IP)

Cells were harvested and then lysed in lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris, 0.05% SDS, 1 mM PMSF, and a 1% cocktail of protease inhibitors) on ice for 20 min. After centrifugation at 4 °C at 13,000 rpm for 10 min, antibodies were added to the supernatant on ice for 1 h. Protein G agarose beads were then added to the samples, and the samples were rotated at 4 °C for 1 h. After the beads were washed three times with lysis buffer, the pellets were dissolved in 2 x SDS loading buffer. Immunoprecipitated protein was analyzed by Western blotting with different antibodies.

2.8. Determination of TrxR enzyme activity

Cells were harvested and resuspended in buffer (50 mM Tris–HCl, pH 7.6 and 1 mM EDTA) and were then sonicated three times for 15-s intervals, followed by centrifugation at 14,000 x g for 10 min at 4 °C. The supernatants were stored at −20 °C for later enzyme activity studies. The activity of TrxR was measured through a coupled reaction in the cell homogenates, essentially as described previously [38].

2.9. Analysis of Trx redox states

For Trx redox analysis, cells were washed once with ice-cold PBS immediately after treatment and quickly lysed in 6 M guanidinium chloride, 50 mM Tris–HCl, pH 8.3, 3 mM EDTA, and 0.5% Triton X-100 containing 50 mM indole acetic acid (IAA) (Sigma) [39]. After 30 min at 37 °C, the excess IAA was removed using Microspin G-25 columns (Amersham Biosciences, Little Chalfont, Bucks., UK), and Trx in different redox states was separated by native PAGE. Western blot analysis was performed to detect the expression of the different redox states using anti-Trx antibody.

2.10. RNA interference assay

A549 cells were plated into 6-wells plate. The following day, siRNAs were transfected using lipofectamine (Invitrogen) at a final concentration of 50 nM. The siRNA directed against TrxR is 5'-AGACACCGCUAUCAUGGGGCAAdTdT-3' and the control is 5'-AGGCAAAUCACGGGUGUCCUdtdTdT-3' [40]. At 72 h after transfection, the cells were harvested, and proteins were extracted for subsequent analysis.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>A549</th>
<th>HCT116</th>
</tr>
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<tbody>
<tr>
<td>Tail area/total area (%)</td>
<td></td>
<td></td>
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<tr>
<td>CTR</td>
<td>24.0 ± 3.9</td>
<td>20.6 ± 4.3</td>
</tr>
<tr>
<td>6 h, 0.1 μM</td>
<td>38.9 ± 14.5</td>
<td>36.9 ± 14.5</td>
</tr>
<tr>
<td>12 h, 0.1 μM</td>
<td>57.5 ± 12.7</td>
<td>68.2 ± 14.3</td>
</tr>
<tr>
<td>CTR</td>
<td>23.8 ± 3.2</td>
<td>22.4 ± 5.3</td>
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<tr>
<td>0.05 μM, 12 h</td>
<td>36.5 ± 8.5</td>
<td>40.9 ± 9.5</td>
</tr>
<tr>
<td>0.1 μM, 12 h</td>
<td>58.5 ± 9.7</td>
<td>65.1 ± 11.3</td>
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<tr>
<td>DNA tail length (μm)</td>
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<tr>
<td>CTR</td>
<td>22.8 ± 7.3</td>
<td>21.7 ± 6.3</td>
</tr>
<tr>
<td>6 h, 0.1 μM</td>
<td>42.5 ± 10.9</td>
<td>51.2 ± 10.3</td>
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<tr>
<td>12 h, 0.1 μM</td>
<td>52.8 ± 13.2</td>
<td>78.9 ± 10.2</td>
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<tr>
<td>CTR</td>
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<td>22.6 ± 9.3</td>
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<td>0.05 μM, 12 h</td>
<td>43.5 ± 17.9</td>
<td>50.2 ± 16.3</td>
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<tr>
<td>0.1 μM, 12 h</td>
<td>94.9 ± 21.2</td>
<td>89.9 ± 11.2</td>
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The presented statistical analysis results from the comet assay in Fig. 1A–D (average mean ± SD of the comet parameters) were measured by CometScore (TriTek Corp., USA) and are shown in this table. The experiments were repeated thrice.

3. Results

3.1. Depsipeptide induces DNA damage

That the HDAC inhibitor depsipeptide alone can activate p53 transactivity [13] raises the possibility that exposure of cells to depsipeptide might induce DNA damage directly or indirectly. To investigate whether depsipeptide causes DNA damage, a comet assay was performed on the human cancer cell lines A549 and HCT116 (both cell lines harbor a wild-type p53). Both cell lines were treated with depsipeptide at 0.05 or 0.1 μM for 6–12 h and were then harvested for the comet assay. In the comet assay, DNA damage is indicated by the presence of a DNA tail, with a greater DNA tail area and longer DNA tail length (distance from the DNA head to the end of the DNA tail) indicating more extensive DNA damage. As shown in Fig. 1A–D, depsipeptide-induced DNA damage was both dose- and duration-dependent in each cell line. The extent of damage was analyzed by software (CometScore), and a statistical analysis of depsipeptide-induced DNA damage is presented in Table 1. To further confirm the depsipeptide induced DNA damage, Western blotting was performed to detect H2AX phosphorylation (γ-H2AX), a well-known marker of DNA damage [41], in both A549 and HCT116 cells with or without depsipeptide treatment. It was also clear that H2AX phosphorylation was significantly increased in depsipeptide treated cells in a dose- and duration-dependent manner when compared to untreated cells (Fig. 1E and F). Compared with depsipeptide, other HDAC inhibitors, such as TSA or sodium butyrate, induced much less DNA damage at the concentration and duration used in this experiment, as demonstrated either by a comet assay or by detection of phosphorylation of H2AX (Fig. 2A–E). These results suggest that depsipeptide plays more broad biological functions than those of other HDAC inhibitors, possibly through its DNA damaging effects.

3.2. Depsipeptide treatment induces ROS generation

The DNA damage induced by many chemical reagents has been reported to be associated with accumulation of ROS [42]. To explore this possibility, ROS generation was detected using the ROS-sensitive fluorescent probe dihydroethidium (DHE). As shown in Fig. 3A–C, we observed elevated ROS generation in A549 cells treated with depsipeptide at 0.05 μM or 0.1 μM for 12 h, with greater effects at 0.1 μM (Fig. 3A–C and F). Similar to findings on DNA damage, TSA and sodium butyrate induced much less ROS generation than did depsipeptide (Fig. 3D–F). To further prove that the depsipeptide-induced generation of ROS is associated with
DNA damage, the free radical scavenger L-N-acetylcysteine (L-NAC) (2.0 mM) was pre-incubated in cell medium to prevent ROS generation. As shown in Fig. 4A–D, depsipeptide-induced ROS generation was significantly decreased in L-NAC pretreated cells. In addition, the H2AX phosphorylation induced by depsipeptide was almost completely blocked by pretreatment with L-NAC (Fig. 4E). Consistent with this result, depsipeptide-induced p53 acetylation at K373/382 and p21 expression were also obviously decreased in L-NAC pretreated cells (Fig. 4E). These data suggest that depsipeptide induced DNA damage and activation of the p53/p21 pathway might result mainly from generation of ROS (Table 2).

3.3. Depsipeptide-induced ROS generation is dependent on inhibition of TrxR expression

Considering that several HDAC inhibitors generate ROS by elevating the expression of non-mitochondrial enzymes [42–44], we measured the expression of several enzymes that generate ROS,
including superoxide dismutase (SOD1 and SOD2), catalase (CAT), thioredoxin (Trx), thioredoxin reductase (TrxR), and glutaredoxin (Grx), in the presence or absence of depsipeptide treatment. With RT-PCR, we did not find obvious changes in SOD, CAT, Trx, or Grx mRNA levels (Fig. 5A). Interestingly, we noticed that the mRNA level of TrxR was significantly decreased after treatment with depsipeptide (Fig. 5A). With Western blotting, we further confirmed the decrease in expression of TrxR after treatment with depsipeptide at 0.1 μM for 12 h (Fig. 5B). Consistent with this result, we also found that the activity of TrxR was remarkably decreased in depsipeptide treated A549 cells (Fig. 5C).

Because TrxR is the only electron donor for Trx, it is possible that Trx might be reversibly inactivated by a change in its redox state. We therefore investigated the effect of depsipeptide on the relative changes in reduced or oxidized Trx in A549 cells. With Western blotting, we observed two bands of Trx in A549 cells treated with depsipeptide, as shown in Fig. 5D. The upper band indicates oxidized Trx, and the lower band indicates reduced Trx [39]. In untreated cells, Trx was mainly observed in the fully reduced thioredoxin state. Treatment with 0.1 μM depsipeptide for 12 h resulted in a significant decrease in the reduced form of Trx (50% decrease in depsipeptide treated sample compared with the control), whereas the oxidized form (upper band) was remarkably increased (more than 2-fold) (Fig. 5D). These data suggest that depsipeptide probably elicits the generation of ROS through oxidation of Trx.

3.4. Depsipeptide-induced DNA damage specifically induces p53 phosphorylation at Thr18 and Ser37

Next, we determined how depsipeptide-induced DNA damage causes p53 acetylation and increases p21 expression. We treated A549 cells with 0.1 μM depsipeptide for 12 h, and proteins were then extracted for Western blotting using anti-phosphorylation antibodies at Ser15, Thr18, Ser20, Ser37 or Ser392, all of which are residues reported to be related to the acetylation of p53 at K373/R382 [30–32,45,46]. As a positive control for p53 phosphorylation, A549 cells were irradiated with gamma rays (8 Gy). As shown in Fig. 6A, ionizing irradiation significantly induced phosphorylation of p53 at all sites tested. Although there were no obvious changes in the phosphorylation of p53 at Ser15, Ser20 or Ser392 in response to depsipeptide treatment, we observed a significant increase in p53 phosphorylation at Thr18 and Ser37 (Fig. 6A), suggesting that depsipeptide might induce p53 phosphorylation with site specificity. To show the generality of these findings, another cell line, HCT116, was also treated with depsipeptide under the same conditions as the A549 cells. As shown in Fig. 6B, depsipeptide also induced obvious changes in p53 phosphorylation at Thr18 and Ser37 in HCT116 cells. Interestingly, both TSA and sodium butyrate were unable to induce phosphorylation of p53 at Thr18 or Ser37 (Fig. 6C), indicating that activation of p53 transactivity is highly specific to depsipeptide.

3.5. p53 phosphorylation at Thr18 is required for p53 acetylation and p21 expression

To further confirm the role of p53 phosphorylation at Thr18 and Ser37 in depsipeptide-induced p53 acetylation at K373/K382 and p21 expression, several p53 mutants were constructed by site-directed mutagenesis of the wild-type p53 expression plasmid. Both Thr18 and Ser37 were mutated to alanine to generate p53 T18A and p53 S37A expression plasmids, and these plasmids were transfected into p53-null H1299 cells. Depsipeptide-induced p53 acetylation and p21 expression were then evaluated. As shown in Fig. 7A, depsipeptide induced a 3.5- or 2.5-fold increase in p53 acetylation at K373/K382 in transfected cells with wild-type p53 or the p53 S37A mutant, respectively. However, p53 acetylation at K373/K382 was not obviously increased in response to depsipeptide treatment when p53T18A was transfected into H1299 cells (Fig. 7A). We also confirmed that p21 expression was impaired in the p53 T18A transfected cells, but not in the wild-type p53 or p53 S37A transfected H1299 cells (Fig. 7A). To further verify the role of p53 phosphorylation at Thr18 in p53 acetylation at K373/K382, a Co-IP assay was performed to test the interaction of p300 and p53. Depsipeptide induced an interaction of p300 and p53, while the p53 T18A mutant impaired the interaction of p300 and p53 (Fig. 7B). To gain more insight into the connection between p53 phosphorylation and activation of the p21 promoter, the wild-type or mutated p53 plasmids were separately transfected into H1299 cells, and a ChIP assay was then performed after depsipeptide treatment. Fig. 7C shows that depsipeptide induced an increase in binding of p53 to the p21 promoter when wild-type p53 or the p53 S37A mutant was transfected into H1299 cells but not when the p53 T18A mutant was transfected into them. This result adds further evidence that phosphorylation of p53 at Thr18 is a prerequisite for
**Fig. 3.** Depsipeptide-induced DNA damage results from ROS generation. A549 cells were treated with depsipeptide at 0 μM as a control (A) and 0.05 μM (B) or 0.1 μM (C) for 12 h. In addition, A549 cells were treated with TSA at 1 μM for 12 h (D) or sodium butyrate at 5 mM for 12 h (E). These treated cells were then stained with DHE for 30 min, and ROS were observed by fluorescence microscopy. Quantification of ROS levels (calculated by fluorescence intensities using confocal microscopy) in cells treated with these HDAC inhibitors was conducted. The relative ratio of ROS accumulation is shown as a histogram (F). Experiments were repeated thrice, and data are presented as means ± SD, *P < 0.05 compared to the control and others HDAC inhibitor treatment.

**Fig. 4.** The ROS scavenger l-NAC blocks depsipeptide-induced accumulation of ROS. A549 cells were treated with PBS as a control (A) or with depsipeptide at 0.1 μM for 12 h in the absence (B) or presence (C) of the ROS scavenger l-NAC (2.0 mM, pretreatment for 2 h). These treated cells were then stained with HE for 30 min, and ROS were observed by fluorescence microscopy. The relative ratio of ROS accumulation is shown as a histogram. Experiments were repeated thrice, and data are presented as means ± SD, *P < 0.05 compared to the control and depsipeptide plus NAC (D). A549 cells were treated with 0.1 μM depsipeptide alone or together with l-NAC for 12 h, and proteins were then extracted for Western blot analysis with anti-γ-H2AX, anti-H3, anti-p21, anti-p53 and anti-Ac373/382-p53 antibodies. We scanned the p53 acetylation bands of Western blots and normalized to the bands of p53-DO-1. The relative changes in p53 acetylation in response to depsipeptide treatment are shown under the panel of p53 acetylation (E). β-Actin was used as a loading control for the Western blot.
p53 acetylation and activation of p21 in response to depsipeptide treatment.

4. Discussion

In the present study, we confirmed that the HDAC inhibitor depsipeptide is a potent inducer of DNA damage. In contrast to other HDAC inhibitors, such as TSA or sodium butyrate, depsipeptide specifically elicited ROS generation by decreasing TrxR expression. In response to depsipeptide-generated ROS, p53 phosphorylation at Thr18 was significantly increased and in turn induced p53 acetylation at lysine 373/382 and p21 expression.

Reactive oxygen species, including superoxide ($O_2^{-}$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($OH^-$), are produced in many ways. Endogenous ROS are primarily generated by incomplete reduction of one-electron oxygen through leakage of electrons from the mitochondrial electron transport chain [47], and exogenous sources of ROS come from several chemical or physical materials, including UV light, $\gamma$-irradiation, and chemical compounds [48–52]. Excess ROS might detrimentally affect cellular functions and result in the damage of proteins, lipids and DNA. To maintain ROS at tolerable levels, several intracellular defense systems exist to scavenge ROS to protect cells from oxidative injury [53]. In mammals, the Trx system, composed of thioredoxin reductase (TrxR), Trx, and NADPH, is a major intracellular defense system against ROS-induced damage [54,55]. An increase in ROS in some cancer cells is associated with a decrease in antioxidants, such as MnSOD and catalase [56–58]. It has been reported that the down-regulation of MnSOD, Trx or Gpx plays a major role in oxidative stress induced by the HDAC inhibitors suberoylanilide hydroxamic acid (SAHA) or sodium butyrate [42,59,60]. Our results provide evidence that, unlike other HDAC inhibitors, depsipeptide mediated-ROS generation results from specifically inhibiting TrxR expression and activity, but not other ROS production related genes such as MnSOD, CAT or Grx [Fig. 5A–C], which is in agreement with a previous report in another cell line [61], indicating that depsipeptide might play an anti-neoplastic role via a unique mechanism. In regard to the mechanism of depsipeptide-induced inhibition of TrxR expression, we suppose that depsipeptide may reduce TrxR expression indirectly, which is not related with DNA demethylation. In our previous published data, depsipeptide-induced DNA demethylation occurred at 48–96 h after treatment [16]. However, in this study, the exposure time of depsipeptide to cells is just 12 h and thus demethylating effect of depsipeptide is not involved in this process in such short time. We do not know the exact mechanism of depsipeptide-induced suppression of TrxR, however, this critical question will be further studied in the future.

As a transcription factor, p53 is often subjected to diverse PTMs such as ubiquitinylation, phosphorylation and acetylation in response to DNA damage [30]. Recent studies have implicated ROS in inducing phosphorylation of p53 [51,52,62]. Generally, p53 phosphorylation at Ser15 and Ser20 has been reported to play a major role in the cellular response to DNA damage, which disturbs the interaction between p53 and its negative regulator, MDM2 [63]. In addition, p53 phosphorylation at Ser392 was reported to be important for inducing growth suppression in response to UV light and non-genotoxic agents [64]. However, in our study, phosphorylation of p53 at Ser15, Ser20 and Ser392 was not observed in the cells in response to depsipeptide treatment (Fig. 6A and B). We found that p53 phosphorylation only at Thr18 and Ser37 was increased after depsipeptide treatment (Fig. 6A and B), a pattern that is different than that observed for PTMs induced by $\gamma$-irradiation or UV light [64,65], but is consistent with other reports that TrxR silencing does not induce phosphorylation of p53 at Ser15 and Ser20, which are the typical PTMs mediated by DNA-damage signaling [66]. We also suppressed TrxR by RNAi techniques and found that
ROS production was increased, by which in turn p53 acetylation and p21 expression were increased (Fig. S2). These results are also consistent with other reports that inhibition of TrxR results in accumulation of oxidized Trx and increased DNA binding activity of p53 and p21 expression [67].

The DNA damage in the cells is sensed by specific kinase(s), p53 can be phosphorylated by numerous kinases, which regulate the specific phosphorylation pattern of p53 in response to different stress. Depending on the types of DNA damage, a specific residue of p53 can be phosphorylated by many kinases, or several residues of p53 can be phosphorylated by a single kinase. The kinases regulating the phosphorylation of p53 at Thr18 include ATM/CHK2 [67], casein kinase 1(CK1) [68, 69], VRK1 (vaccinia-related kinase 1) [70], DNA-PK [71] and VRK2 (vaccinia-related kinase 2) [72]. However, phosphorylation of p53 at Ser37 is mainly carried out by ATR [73] and DNA-PK [74].

It has been demonstrated that oxidative stress (H₂O₂ or other peroxide) can trigger the ATM-mediated DNA damage signaling pathway [75, 76]. In addition, ATM has been considered to be an oxidative stress sensor [77]. Based on these reports mentioned above, ATM-dependent pathway might be predominant in response to depsipeptide-induced DNA damage, which is consistent with other reports [11] and our results (Fig. S2).

In the present study, we detected the DNA damage by the alkaline comet assay. Generally, the neutral comet assay is for detecting DNA double strand breaks, whereas the alkaline comet is for detecting single and double strand breaks [36, 78]. The results of the present study indicate that depsipeptide may result in double-strand breaks.

It is clear in this study that p53 phosphorylated at Thr18 only plays an important role in depsipeptide induced p21 activation (Fig. 7A and C). Several studies have shown that in response to stress, the activity of p53 is regulated by multiple modifications, which might be coordinated or might be interdependent [31, 34, 35]. Consistent with these previous studies, we also show that depsipeptide activates p21 expression through p53 phosphorylation specifically at Thr18 and subsequent acetylation at K373/K382 in this study (Fig. 7A). It is possible that p53 phosphorylation at Thr18 might be a prerequisite for p53 acetylation at K373/382 in response to ROS-induced DNA damage. Furthermore, this activation of p53 by a phosphorylation–acetylation cascade is consistent with previous reports. For example, DNA damage mediated phosphorylation of p53 at Thr18 promotes interaction between p53 and CBP/p300 or PCAF, through which p53 is acetylated at specific sites [79]. Recently, biophysical analysis has shown that, among phosphorylation of p53 residues (Ser15, -20, -33, -37, -46, -55 and Thr18) in
response to various stimuli, Thr18 phosphorylation has the most potent impact on the p53/p300 interaction [80,81].

In our study, p53 phosphorylation at Ser37 is not required for p53 acetylation at K373/382 and p21 expression (Fig. 7A and C). This result is inconsistent with a previous report [31]. We consider that p53 Ser37 might cooperate with other modifications to activate p21 in response to certain stimuli. Depsipeptide-induced DNA damage results from generation of ROS and thus might induce a different range of DNA damage responses from that of damage induced by ionizing radiation or other DNA damage inducers [35,64]. However, this specificity of depsipeptide-induced p53 modifications and changes in p53 function are helpful for us to understand the complexity of p53 posttranslational modifications in response to different types of DNA damage. In addition, these data will provide guidance for the clinical application of depsipeptide.

Conflict of interest

The authors declare that there are no conflicts of interest for this study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dnarep.2011.10.014.


