Inhibition of Histone Deacetylases by Chlamydocin Induces Apoptosis and Proteasome-Mediated Degradation of Survivin

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ABSTRACT

The naturally occurring cyclic tetrapeptide chlamydocin is a very potent inhibitor of cell proliferation. Here we show that chlamydocin is a highly potent histone deacetylase (HDAC) inhibitor, inhibiting HDAC activity in vitro with an IC50 of 1.3 nM. Like other HDAC inhibitors, chlamydocin induces the accumulation of hyperacetylated histones H3 and H4 in A2780 ovarian cancer cells, increases the expression of p21cip1/waf1, and causes an accumulation of cells in G2/M phase of the cell cycle. In addition, chlamydocin induces apoptosis by activating caspase-3, which in turn leads to the cleavage of p21cip1/waf1 into a 15-kDa breakdown product and drives cells from growth arrest into apoptosis. Concomitant with the activation of caspase-3 and cleavage of p21cip1/waf1, chlamydocin decreases the protein level of survivin, a member of the inhibitor of apoptosis protein family that is selectively expressed in tumors. Although our data indicate a potential link between degradation of survivin and activation of the apoptotic pathway induced by HDAC inhibitors, stable overexpression of survivin does not suppress the activation of caspase-3 or cleavage of p21cip1/waf1 induced by chlamydocin treatment. The decrease of survivin protein level is mediated by degradation via proteasomes since it can be inhibited by specific proteasome inhibitors. Taken together, our results show that induction of apoptosis by chlamydocin involves caspase-dependent cleavage of p21cip1/waf1, which is strikingly associated with proteasome-mediated degradation of survivin.

Histone acetylation and deacetylation are believed to play a key role in the regulation of gene transcription. The histone acetylation levels are tightly controlled by the dynamic equilibrium between competing histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs are recruited by transcription factors and are associated with activation of transcription, whereas HDACs are involved in transcriptional silencing (Kuo and Allis, 1998). To date, at least 18 different mammalian HDACs have been described and are divided into three classes. Class I is related to yeast Rpd3 and contains HDAC1, HDAC2, HDAC3, HDAC8, and the recently cloned HDAC11 (Gray and Ekstrom, 2001; Gao et al., 2002). Class II is related to yeast Hda1 and contains HDAC4 (= A), HDAC5 (= B), HDAC6, HDAC7, and the recently cloned HDAC9 and HDAC10 (Gray and Ekstrom, 2001; Kao et al., 2001; Zhou et al., 2001); and class III is the silent information regulator 2 (Sir2)-like family in which the HDAC activity depends upon nicotinamide-adenine dinucleotide and contains sirtuins (Sir2) 1 to 7 (Frye, 2000; Imai et al., 2000).

HATs and HDACs play a key role in normal cell cycle progression and differentiation (Kouzarides, 1999). It is therefore not surprising that aberrant acetylation has been linked to cellular transformation, suggesting that both HATs and HDACs play an important role in carcinogenesis (Cress and Seto, 2000; Marks et al., 2001). This hypothesis is further strengthened by the fact that small-molecule inhibitors of HDACs have been shown to induce cell cycle arrest, differentiation, and apoptosis, suggesting that they might be promising anticancer drugs (Marks et al., 2001; Vigushin and Coombes, 2002). However, the exact molecular mechanisms underlying these effects are poorly understood.

Several HDAC inhibitors have been reported to increase the expression of p21cip1/waf1 (Vigushin and Coombes, 2002). Furthermore, a number of these inhibitors have been shown to induce apoptosis (Marks et al., 2001). The induction of

ABBREVIATIONS: HAT, histone acetyltransferase; HDAC, histone deacetylase; Z-VAD-fmk, benzylloxy carbonyl-Val-Ala-Asp(O-Me)-fluoromethyl ketone; Z-DEVD-fmk, benzylloxy carbonyl-Asp-Glu(OMe)-Val-Asp(O-Me)-fluoromethyl ketone; MG132, Z-Leu-Leu-Leu-aldehyde; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; EGFP, enhanced green fluorescent protein; tTA, tetracycline controlled transcriptional activator.
apoptosis is associated with activation of caspase-3 (Medina et al., 1997). Activation of caspase-3 results in the cleavage of a number of downstream substrates, including p21cip1/waf1 (Ambrosini et al., 1998; Li et al., 1999). Caspase-dependent cleavage of p21cip1/waf1 is a critical step that drives cancer cells from growth arrest into apoptosis (Zhang et al., 1999).

During mitosis, p21cip1/waf1 and caspase-3 have been shown to be localized to the mitotic spindle where they colocalize with survivin (Li et al., 1999). Survivin is a recently described member of the inhibitor of apoptosis protein (IAP) family (Ambrosini et al., 1997) that is selectively expressed in practically all of the most common human cancers but not in adjacent normal cells (Ambrosini et al., 1997). Survivin is expressed in a cell cycle-dependent manner, and its expression is increased during G2/M phase of the cell cycle (Li et al., 1998). It is hypothesized that survivin plays a critical role in the p21cip1/waf1/caspase-3 complex by directly inhibiting caspase activity and preventing cells from undergoing apoptosis during normal cell division (Li et al., 1999). Indeed, it has been shown that decreased survivin protein levels result in induction of apoptosis, characterized by caspase-dependent cleavage of p21cip1/waf1 (Ambrosini et al., 1998; Li et al., 1999; Chen et al., 2000).

Chlamydocin (Fig. 1) was originally isolated from the fungus Diheterospora chlamydospora and has been shown to exhibit potent anticancer activity in vitro (Closee and Huguenin, 1974). Chlamydocin belongs to a small family of hydrophobic cyclic tetrapeptides containing the unusual amino acid, 2-amino-8-oxo-9,10-epoxy decanoic acid (Aoe), which is essential for their biological activity. Chlamydocin has already been characterized as a highly potent inhibitor of mammalian HDAC activity (Furumai et al., 2001).

In this study, we further characterized chlamydocin as a highly potent HDAC inhibitor and studied the molecular mechanisms underlying chlamydocin-induced apoptosis.

**Materials and Methods**

**Materials**

Chlamydocin, trichostatin A, and MG132 were purchased from Calbiochem (La Jolla, CA). Lactacyclin was purchased from Sigma-Aldrich (St. Louis, MO), and Z-VAD-fmk and Z-DEVD-fmk were obtained from Kamiya Biomedical Company (Seattle, WA). All compounds were dissolved in dimethyl sulfoxide, and aliquots were stored at -20°C. Compounds were diluted in cell culture medium just before use.

**Cell Culture**

Human A2780 ovarian carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). They were grown in RPMI 1640 cell culture medium supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) glutamine (200 mM), and 1% (v/v) gentamicin (5 mg/ml) (all reagents were obtained from Invitrogen, Carlsbad, CA). A549.tTA#21, a human lung carcinoma cell line engineered to overexpress the tetracycline-controlled transactivator required for tet-regulatable expression vectors (Y. Hitoshi and D. Payan, unpublished data), was kindly provided by Rigel Pharmaceuticals Inc. (South San Francisco, CA). Cells were grown in F12-K cell culture medium supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (all reagents were obtained from Invitrogen). Human HeLa cervical carcinoma cells (American Type Culture Collection) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated (30 min at 56°C) fetal bovine serum, 1% (v/v) glutamine (200 mM), 1% (v/v) sodium pyruvate (200 mM), and 1% (v/v) gentamicin (5 mg/ml) (all reagents were obtained from Invitrogen). Human H1299 lung carcinoma cells (American Type Culture Collection) were grown in RPMI 1640 cell culture medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 1% (v/v) glutamine (200 mM), 1% (v/v) sodium pyruvate (200 mM), and 1% HEPES (1 M) (Cambrex, Ver- viers, Belgium).

Phoenix amphotropic packaging cells, kindly provided by Rigel Pharmaceuticals Inc. (South San Francisco, CA), were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

**Preparation of Total Cell Lysates and Western Blot Analysis**

Subconfluent cells were treated with indicated concentrations of chlamydocin or trichostatin A for the indicated periods of time. Thereafter, both floating and adherent cells were collected and total cell lysates were prepared by lysis in a buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM dithiothreitol, 0.1% (v/v) Tween 20, 10% (v/v) glycerol, and protease inhibitors (complete Mini EDTA-free protease inhibitor cocktail tablets; Roche Diagnostics, Mannheim, Germany). Cells were lysed by passage through a 22-gauge needle (BD Biosciences, Fraga, Spain), two cycles of freeze-thawing, and sonication during 10 s. After centrifugation, protein concentration in the supernatant was determined using Coomassie Plus Protein Assay Reagent (Pierce Chemi- cal, Rockford, IL). Proteins were separated by SDS-polyacrylamide gel electrophoresis on either 8% or 16% Tris-glycine gel and transferred to an Immob-Blot polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA) by semidyly blotting. Hyperacetylated histones were detected using antibodies that specific-ly recognize hyperacetylated forms of histone H3 or histone H4 (Upstate Biotechnology, Lake Placid, NY). p21cip1/waf1 protein was detected by use of an antibody that recognizes p21cip1/waf1 and its cleavage product p15 (BD Biosciences PharMingen, San Diego, CA). Caspase-3 was detected using an antibody from Research Diagnos- tics (Flanders, NJ) and survivin using an antibody raised against full-length recombinant human survivin (Novus Biologicals, Littleton, CO). Horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, and Zymed Labarato-
ries, South San Francisco, CA) were used. Protein-antibody complexes were then visualized by chemiluminescence according to the manufacturer’s instructions (Pierce Chemical). As a control for equal amounts of protein loading, an antibody against actin was used (Oncogene Research Products, San Diego, CA).

**HDAC Activity Assay**

Total cell lysates from A2780 cells were preincubated for 10 min at room temperature with increasing concentrations of chlamydocin (0.01 nM to 1 μM). Histone deacetylase activity was measured by incubating 3 μg of total cell lysate with a [3H]acetyl-labeled fragment of histone H4 peptide (~50,000 cpm) (Amersham Biosciences, Piscataway, NJ) and HDAC buffer (25 mM HEPES pH 7.4, 1 μM sucrose, 0.1 mg/ml bovine serum albumin, and 0.01% v/v Triton X-100) in 40 μl for 30 min at room temperature. Assays were performed in quadruplicate. After incubation, the reaction was quenched with 35 μl of stop buffer (1 M HCl and 0.4 M acetic acid). Released [3H]acetic acid was extracted with 800 μl of ethyl acetate and quantified by scintillation counting. Results are presented as mean ± standard deviation of three independent experiments. IC$_{50}$ values were calculated by nonlinear regression analysis using SigmaPlot 4.01 software (SPSS Science, Chicago, IL).

**Proliferation Assays**

The effect of chlamydocin on cell proliferation was measured using an MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide]-based assay (Serva, Heidelberg, Germany) in 96-well plates. Cells were treated with different concentrations of chlamydocin. After 4 days of treatment, culture medium was renewed (200 μl) and 25 μl of MTT (5 mg/ml in phosphate-buffered saline) solution was added, and cells were further incubated for 2 h in a cell incubator. Thereafter, the medium was aspirated. Twenty-five microliters of Sorenson glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) were added together with 100 μl of dimethyl sulfoxide to solubilize the blue MTT-formazan product. Absorbance was measured at 540 nm using a spectrophotometer. Data are presented as mean ± standard deviation of three independent experiments. Within an experiment, the result of each experimental condition is the mean of six replicate wells. The IC$_{50}$ values were calculated by nonlinear regression analysis using SigmaPlot 4.01 software.

The effect of chlamydocin on A2780 cell proliferation was further investigated using trypan blue exclusion. Cells were treated with 1 μM chlamydocin. After 24, 48, and 72 h of treatment, both floating and adherent cells were collected and stained with trypan blue, and both viable and dead cells were counted with a Cedex cell counter (Applitek, Deinze, Belgium). Data are presented as mean ± standard deviation of three independent experiments. Within an experiment, the result of each experimental condition is the mean of 20 counts.

**Detection of Apoptosis**

**Flow Cytometry.** Apoptosis was detected using the Annexin-V-FLUOS Staining Kit from Roche Diagnostics. A2780 cells were cultured with 1 μM chlamydocin for the indicated periods of time. Thereafter, cells were harvested and stained with annexin V and propidium iodide according to the manufacturer’s instructions. Analysis was performed on a FACScan flow cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA).

**Immunohistochemistry.** Both floating and adherent cells were collected, centrifuged, washed in Hanks’ balanced salts solution, and fixed with 1% paraformaldehyde. An artificial tissue was made by treating the cell pellets with human plasma serum and thrombin. The artificial tissue was then fixed in paraformaldehyde and embedded in paraffin. Then, sections were deparaffinized, rehydrated, and treated with H$_2$O$_2$ to block endogenous peroxidase. After a microwave citrate pretreatment, the samples were incubated with the primary antibody, anti-cleaved poly(ADP-ribose) polymerase (Promega Corporation, Leiden, The Netherlands). Peroxidase-labeled secondary antibodies were used. Protein-antibody complexes were then visualized using 3-amino-9-ethylcarbazole. For nuclear counterstaining, hematoxylin was used. As a negative control, the primary antibody was omitted in the staining procedure.

**Plasmids**

**Survivin/pRTIG.** the full-length coding sequence of human survivin cDNA (corresponding to European Molecular Biology Laboratory accession number U75825) was amplified by polymerase chain reaction and subcloned into the TTA retroviral expression vector (Lorena et al., 2000). This vector contains a tetracycline-regulatable expression cassette and a self-inactivating mutation in the 3′ long terminal repeat, allowing the TRE-CMV I/E (tetracycline-responsive element-cytomegalovirus immediate/early) promoter to drive expression of the insert without influence of the long terminal repeat enhancer elements. A bicistronic mRNA was transcribed from this vector, encoding survivin and enhanced green fluorescent protein (EGFP). Translation of the latter was initiated via an internal ribosomal entry site, inserted between the survivin and the EGFP open reading frame.

**Retroviral Transduction**

Retroviral transduction was performed as described (Swift et al., 1999) using the spin infection protocol. Briefly, amphotropic Phoenix cells were transfected with survivin/pRTIG, tTA/pRCIH, empty pRIG vector, or combinations thereof. After 6 to 8 h, transfection medium was replaced by fresh medium. Sixteen hours later, medium was replaced by A2780 or A549.tTA#21 culture medium, and cells were incubated at 32°C for 24 h. The supernatant was aspirated and added to A2780 or A549.tTA#21 cells plated in six-well plates at a density of 3 × 10$^5$ and 1 × 10$^6$ cells per well, respectively. Cells were incubated at 37°C for 24 h. Finally, the retroviral supernatant was replaced by fresh medium. Transduced cells were analyzed and sorted with a MoFlo fluorescence-activated cell sorter (Cytomation, Fort Collins, CO), and cells showing highest expression of EGFP (upper 10%) were recovered.

**Results**

**Chlamydocin Is a Potent Inhibitor of Cell Proliferation.** Chlamydocin (Fig. 1), a fungal metabolite, has been shown to exhibit potent antiproliferative activity in vitro (Closse and Huguenin, 1974). The effect of chlamydocin was examined on several human cancer cell lines with different p53 status. As shown in Table 1, chlamydocin potently inhibited the proliferation of all the tested cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>IC$_{50}$ nM</th>
</tr>
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<tbody>
<tr>
<td>A2780</td>
<td>Ovarian</td>
<td>0.36 ± 0.15</td>
</tr>
<tr>
<td>Malme-3M</td>
<td>Melanoma</td>
<td>45 ± 2.5</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Breast</td>
<td>5.3 ± 2.3</td>
</tr>
<tr>
<td>HT-29</td>
<td>Colon</td>
<td>4.3 ± 0.8</td>
</tr>
<tr>
<td>HeLa</td>
<td>Cervix</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>H1299</td>
<td>Lung</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>K562/C1000</td>
<td>Leukemia</td>
<td>0.76 ± 0.06</td>
</tr>
</tbody>
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**TABLE 1**

Chlamydocin inhibits cell proliferation in various cell lines: MTT-based assay as described under Materials and Methods.
The antiproliferative activity of chlamydocin was further examined by counting A2780 cells treated with chlamydocin using trypan blue exclusion. A2780 cells were treated with 1 μM chlamydocin for 24, 48, and 72 h. As shown in Table 2, 1 μM chlamydocin induced growth inhibition after 24 h. At later time points, cell death occurred. In addition, flow cytometric analysis was performed. A2780 cells were treated with 1 μM chlamydocin for the indicated periods of time. As shown in Fig. 2, chlamydocin induced accumulation of cells in G2/M and a concomitant decrease of cell population in G1 and S phase. After 24 h, chlamydocin induced apoptosis, as shown by the appearance of a sub-G1 cell population peak. In addition, the induction of apoptosis was accompanied by the appearance of floating cells in the culture medium. The floating cell population showed massive annexin V staining (propidium iodide negative), a feature characteristic of apoptosis (data not shown).

Chlamydocin Is a Potent Histone Deacetylase Inhibitor. Since chlamydocin is structurally related to trapoxin A (Fig. 1), a well known histone deacetylase inhibitor (Sambucetti et al., 1999), the effect of chlamydocin on histone deacetylase activity was examined using A2780 total cell lysates in vitro. As previously reported, chlamydocin inhibits histone deacetylase activity (Furumai et al., 2001). As shown in Fig. 3A, we have reproduced these observations and report that chlamydocin inhibited histone deacetylase activity in a concentration-dependent manner with a calculated IC50 value of 1.3 ± 0.5 nM. This potency is comparable with that of trichostatin A (Fig. 1) under similar conditions (IC50 ~2 nM, data not shown).

To examine the effect of chlamydocin on intact cells, the acetylation status of histone H3 and H4 was determined after treatment for 4 h with different concentrations of chlamydocin. Figure 3B shows that chlamydocin induced both histone H3 and histone H4 hyperacetylation in a concentration-dependent manner. Histone hyperacetylation became apparent at a concentration of 1 nM but reached its maximal level at a concentration of 10 nM. The induction of histone hyperacetylation was further examined using 1 μM chlamydocin at different times (Fig. 3C). The accumulation of hyperacetylated histones H3 and H4 was very rapid and already detectable after 2 h of treatment. Levels of hyperacetylated histones H3 and H4 increased further and persisted up to 24 h.

Chlamydocin Induces Apoptosis. Histone deacetylase inhibitors have been shown to induce p21cip1/waf1 expression (Vigushin and Coombes, 2002). The effect of chlamydocin on p21cip1/waf1 protein expression in A2780 lung carcinoma cells was determined by Western blot analysis. Chlamydocin increased the expression of p21cip1/waf1 already after 6 h (Fig. 4). In addition, after 12 h, a p21cip1/waf1-derived cleavage product of ~15 kDa (p15) (Chai et al., 2000a) was detected. The appearance of p15 was concentration-dependent and predominantly present in the floating (apoptotic-rich) cell population (Fig. 5A). In contrast, in adherent (viable) cells, p15 was nearly undetectable. Also, in HeLa cervix carcinoma cells, chlamydocin induced the appearance of p15 in the floating cell population. Furthermore, the structurally unrelated HDAC inhibitor trichostatin A (Fig. 1) also induced p15 in a concentration-dependent manner (Fig. 5B).

The appearance of the p15 cleavage product occurred concomitantly with the activation of caspase-3 (Fig. 4). Activation of caspase-3 was measured using an antibody that specifically recognizes the 17-kDa subunit that is derived from cleavage of the proenzyme form of caspase-3 in cells undergoing apoptosis. Chlamydocin-induced cleavage of p21cip1/waf1 into p15 was inhibited by the caspase peptide inhibitors Z-VAD-fmk and Z-DEVD-fmk (Fig. 5C).

Chlamydocin Induces Proteasome-Mediated Degradation of Survivin. To further explore the molecular mechanisms underlying the induction of apoptosis induced by chlamydocin, we monitored the expression of survivin. A2780 cells were treated with 1 μM chlamydocin for the indicated periods of time (Fig. 4). Chlamydocin decreased the level of survivin protein expression after 12 h as measured by densitometric analysis. The decrease in survivin protein levels occurred concomitantly with activation of caspase-3 and cleavage of p21cip1/waf1 (Fig. 4). Since no decrease of survivin mRNA was detected (data not shown) and since it is known that survivin degradation is tightly regulated via the ubiquitin-proteasome pathway (Zhao et al., 2000), it was determined whether chlamydocin-induced degradation of survivin occurred via proteasomes. As shown in Fig. 6A, chlamydocin treatment for 24 h clearly decreased survivin protein levels. Both proteasome inhibitors, lactacystin and MG132, had no effect by themselves, but were able to inhibit chlamydocin-induced degradation of survivin (Fig. 6A).

Degradation of Survivin Is Not the Main Trigger for Induction of Apoptosis by Chlamydocin. To examine whether survivin degradation was the main trigger for chlamydocin-induced apoptosis, A2780 ovarian carcinoma
and A549 lung carcinoma cell lines were generated stably overexpressing survivin under a tet-regulatable promoter. In vector-infected A2780 control cells, chlamydocin induced apoptosis via activation of caspase-3 and cleavage of p21<sup>cip1/waf1</sup> (Fig. 6B). Upon withdrawal of doxycycline in survivin-infected A2780 cells, survivin was clearly overexpressed as compared with vector-infected cells (Fig. 6B). Under these conditions, despite the marked overexpression of survivin, chlamydocin continued to induce massive apoptosis, which was accompanied by activation of caspase-3 and cleavage of p21<sup>cip1/waf1</sup>. A decrease in survivin protein level induced by chlamydocin could not be detected, most likely due to massive overexpression of survivin. Upon addition of doxycycline to survivin-overexpressing A2780 cells, survivin levels returned to more normal levels and the chlamydocin-induced decrease of survivin protein levels was detectable again (Fig. 6B). Essentially identical results were obtained in A549 cells stably overexpressing survivin (data not shown). The apoptotic activity of chlamydocin was further examined with immunohistochemistry, detecting poly(ADP-ribose) polymerase cleavage. As shown in Fig. 7, chlamydocin induced apoptosis when survivin protein levels were basal (Fig. 7B) or suppressed to more normal levels using doxycycline in survivin-overexpressing A2780 cells (Fig. 7D). Also, under conditions where survivin was clearly overexpressed (Fig. 7C), chlamydocin was still able to induce apoptosis. Taken together, these data indicate that survivin overexpression was unable to inhibit chlamydocin-induced apoptosis.

**Discussion**

In this study, we have characterized the naturally occurring cyclic tetrapeptide chlamydocin, a known anticancer agent, as a highly potent HDAC inhibitor. In addition, the molecular mechanisms through which chlamydocin induces apoptosis were studied. We have shown that chlamydocin-induced apoptosis involves caspase-dependent cleavage of...
We have also shown that chlamydocin is a highly potent HDAC inhibitor, inhibiting HDAC activity in vitro with a calculated IC$_{50}$ of 1.3 nM, in agreement with data from Furumai et al. (2001). Chlamydocin is a much more potent inhibitor of mammalian HDACs as compared with HDACs purified from plants, where micromolar concentrations of chlamydocin were needed to significantly inhibit HDAC activity (Brosch et al., 1995).

Chlamydocin exhibits a broad spectrum of antiproliferative activity toward various cancer cell lines, irrespective of their p53 status. The antiproliferative activity of chlamydocin was accompanied by accumulation of hyperacetylated histones H3 and H4, induction of p21$^{cip1/waf1}$, and accumulation of cells in G$_2$/M phase of the cell cycle. These results are similar to studies with other HDAC inhibitors (Marks et al., 2001; Vigushin and Coombes, 2002), underscoring the fact that chlamydocin is a genuine HDAC inhibitor. In addition, chlamydocin is structurally related to trapoxin A, a known HDAC inhibitor that induces apoptosis in A549 lung carcinoma cells (Sambucetti et al., 1999).

We have shown that chlamydocin induces apoptosis in A2780 ovarian cancer cells. The induction of apoptosis was accompanied by the appearance of floating cells with sub-G$_1$ DNA content, positive annexin V staining, negative propidium iodide staining, and activation of caspase-3, features that are characteristic of apoptosis (Longthorne and Williams, 1997; Darzynkiewicz and Bedner, 2000). Other HDAC inhibitors have also been shown to induce apoptosis (Medina et al., 1997; Sambucetti et al., 1999; Vigushin and Coombes, 2002), but the underlying molecular mechanisms are poorly understood. It has been shown that induction of apoptosis by trichostatin A requires protein synthesis and leads to the activation of caspase-3 protease activity (Medina et al., 1997). Chlamydocin clearly activated caspase-3 activity in a time-dependent manner. In addition, a caspase-dependent cleavage product of p21$^{cip1/waf1}$ was detected that was only present in the apoptotic cell population. Recently, caspase-dependent cleavage of p21$^{cip1/waf1}$ during apoptosis has been reported for a number of apoptotic stimuli, such as tumor necrosis factor (Donato and Perez, 1998), growth factor deprivation (Levkau et al., 1998), DNA damage (Gervais et al., 1998; Zhang et al., 1999), and butyrate treatment (Chai et al., 2000a). Induction of apoptosis by the short-chain fatty acid butyrate has been suggested to involve inhibition of HDAC activity (Chai et al., 2000a). However, millimolar concentrations are needed to achieve these effects, and at these concentrations, sodium butyrate also interferes with other molecular pathways (Kruh, 1982; Gibson, 2000). Our results, obtained with the highly potent HDAC inhibitor chlamydocin, clearly illustrate that the induced apoptosis is accompanied by cleavage of p21$^{cip1/waf1}$. The fact that the structurally unrelated HDAC inhibitor trichostatin A also induced apoptosis, which involved cleavage of p21$^{cip1/waf1}$, suggests that this might be a general effect shared by HDAC inhibitors.

Since the potent induction of p21$^{cip1/waf1}$ expression is believed to play a pivotal role in mediating the antiproliferative effects of HDAC inhibitors (Marks et al., 2001; Vigushin and Coombes, 2002), the cleavage of p21$^{cip1/waf1}$ seems to play a crucial role in converting cells from growth arrest to undergoing apoptosis (Zhang et al., 1999).

To further explore the molecular mechanisms that lead to cleavage of p21$^{cip1/waf1}$, the protein levels of survivin were examined. Survivin is a recently described member of the inhibitor of apoptosis protein family (Ambrosini et al., 1997), which is selectively expressed in all of the most common human cancers but not in adjacent normal cells (Ambrosini et al., 1997). Survivin is expressed in a cell cycle-dependent manner, and its expression is increased during G$_2$/M phase of the cell cycle (Li et al., 1998). Surprisingly, chlamydocin treatment strongly reduced the protein levels of survivin even though cells accumulated in G$_2$/M phase. Since the down-regulation of survivin occurred concomitantly with p21$^{cip1/waf1}$ cleavage and caspase-3 activation, these results suggested that survivin down-regulation was a major trigger for chlamydocin-induced apoptosis. Indeed, survivin had been characterized as a direct and potent inhibitor of caspase-3 (Shin et al., 2001), and it had been suggested that survivin keeps the caspases in an inhibitory state. In this way, survivin could protect p21$^{cip1/waf1}$ from proteolytic cleavage by caspase-3, which was also suggested by results obtained by survivin down-regulation using antisense oligodeoxynucleotides (Ambrosini et al., 1998; Li et al., 1999; Chen et al., 2000). In clear contrast, our results showed that stable overexpression of survivin was unable to protect against chlamydocin-induced apoptosis. These data imply that survivin is unable to directly suppress caspase activity in intact cells, which is in contrast with its ability to directly inhibit caspases in vitro (Donato and Perez, 1998). On the other hand, Suzuki et al. (2000) also suggested that survivin does not directly suppress caspase-3 in cells because overexpressed survivin only partially prevented Fas-mediated cell death. Clearly, further research is necessary to clarify the
controversy surrounding the exact function and mechanism of action of survivin (Banks et al., 2000; Conway et al., 2000). Our data may also have implications for the use of HDAC inhibitors in clinical trials. They indicate that HDAC inhibitors are very potent inducers of apoptosis despite strong overexpression of survivin, illustrating the potential value of these compounds for cancer therapy (Marks et al., 2001; Vigushin and Coombes, 2002).

Survivin degradation is tightly regulated via the ubiquitin-proteasome pathway (Zhao et al., 2000). Here we showed that chlamydacin-induced degradation of survivin via the ubiquitin-proteasome pathway. Both proteasome inhibitors MG132 and lactacystin prevented chlamydacin-induced degradation of survivin. This effect appeared to be quite specific for survivin since chlamydacin-induced down-regulation of XIAP, another member of the IAP family, did not occur via the proteasome pathway (S. De Schepper and J. Van heusden, unpublished data). The mechanism(s) by which chlamydacin activates the proteasome pathway are currently unknown. Furthermore, ubiquitination of survivin has been shown to occur at several lysine residues (Zhao et al., 2000). It is therefore tempting to speculate that survivin degradation may be mediated via acetylation of specific lysine residues.

In summary, in this study we characterized chlamydacin as a highly potent HDAC inhibitor and we showed that chlamydacin-induced apoptosis involves caspase-dependent cleavage of p21<sup>15-kDa</sup> and caspase-3. Strikingly, concomitant proteasome-mediated degradation of survivin was also observed. Further work will be necessary to explore additional mechanisms that lead to induction of apoptosis and to activation of the proteasome pathway.
Ambrosini G, Adida C, Sirugo G, and Altieri DC (1998) Induction of apoptosis and
Ambrosini G, Adida C, and Altieri DC (1997) A novel anti-apoptosis gene, survivin,

Fig. 7. Effect of chlamydocin on apoptosis, visualized by immunohisto-

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