Effects of the antifungal agent ciclopirox in HPV-positive cancer cells: Repression of viral E6/E7 oncogene expression and induction of senescence and apoptosis

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The malignant growth of human papillomavirus (HPV)-positive cancer cells is dependent on the continuous expression of the viral E6/E7 oncogenes. Here, we examined the effects of iron deprivation on the phenotype of HPV-positive cervical cancer cells. We found that iron chelators, such as the topical antifungal agent ciclopirox (CPX), strongly repress HPV E6/E7 oncogene expression, both at the transcript and protein level. CPX efficiently blocks the proliferation of HPV-positive cancer cells by inducing cellular senescence. Although active mTOR signaling is considered to be critical for the cellular senescence response towards a variety of prosenescent agents, CPX-induced senescence occurs under conditions of severely impaired mTOR signaling. Prolonged CPX treatment leads to p53-independent Caspase-3/7 activation and induction of apoptosis. CPX also eliminates HPV-positive cancer cells under hypoxic conditions through induction of apoptosis. Taken together, these results show that iron deprivation exerts profound antiviral and antiproliferative effects in HPV-positive cancer cells and suggest that iron chelators, such as CPX, possess therapeutic potential as HPV-inhibitory, pro-senescent and proapoptotic agents in both normoxic and hypoxic environments.

Introduction
About 5% of the total human cancer incidence is attributable to infections with oncogenic types of human papillomaviruses (HPVs) which includes prevalent cancers in the anogenital region and of the oropharynx. The viral E6 and E7 oncoproteins play a central role in the oncogenicity of HPVs. Both are pleiotropic factors that modulate the function of a broad variety of cellular proteins and pathways. The inactivation of the p53 and pRb tumor suppressor proteins by E6 and E7, respectively, plays a central role for HPV-linked transformation. Under many experimental conditions, the combined inhibition of E6/E7 expression leads to a rapid induction of cellular senescence, an irreversible growth arrest. This indicates that HPV-positive cancer cells are “oncogene addicted” in that they require continuous E6/E7 expression for their proliferation. Thus, it will be important to identify regulatory mechanisms that can block E6/E7 expression and thereby possess the potential to be developed into new treatment options for HPV-positive (pre)neoplastic lesions.

Cancer cells typically exhibit metabolic alterations, which contribute to their malignant growth and to their resistance towards anticancer therapies. A classic example of metabolic reprogramming is the increased rate of aerobic glycolysis in cancer cells (Warburg effect) which provides building blocks necessary for cell proliferation, such as nucleotides, amino acids and lipids. More recently, changes in iron metabolism have also been linked to carcinogenesis. In specific, tumor cells typically reprogram various cellular processes that ultimately lead to enhanced iron influx and reduced iron efflux. The resulting increase in the intracellular iron pool could exert protumorigenic effects, for example, by supporting DNA synthesis and cell proliferation or by affecting cancer-
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also can target hypoxic HPV-positive cancer cells, which are typi-
conditions of impaired mTOR (mechanistic target of rapamycin)
conditions. Notably, CPX-induced senescence can occur under
tion of cellular senescence or apoptosis, depending on treatment
growth inhibition of HPV-positive cancer cells through the induc-
ably more resistant towards radiotherapy and chemotherapy,14
show that HPV oncogene expression and the growth of HPV-
positive cells are highly sensitive to iron deprivation and indicate
leading to induction of apoptosis. Collectively, these
findings show that HPV oncogene expression and the growth of HPV-
positive cancer cells are highly sensitive to iron deprivation and indicate
that CPX may possess therapeutic potential as an antiviral, pro-
senescence and/or proapoptotic agent in both normoxic and hypoxic
HPV-positive cancer cells.

Materials and Methods
Cell culture and treatment conditions
HPV18-positive HeLa (RRID:CVCL_0030) and HPV16-positive
SiHa (RRID:CVCL_0032) cervical cancer cells were obtained from
the tumor bank of the German Cancer Research Center
(DKFZ), Heidelberg. HeLa “p53 null” cervical cancer cells
were generated as described.15 These cells were cultivated at 37°C in
DMEM (Gibco, Life Technologies, Carlsbad, CA) containing
10% fetal bovine serum (Gibco, Life Technologies), 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma-
Aldrich, St. Louis, MO) at 5% CO2 and 21% O2 (normoxia).
HPV-negative HCT116 (RRID:CVCL_0291) and HCT116 p53−/−
(RRID:CVCL_HD97)16 colon cancer cells were obtained from
Dr. Bert Vogelstein (Johns Hopkins, Baltimore, MD) and culti-
vated in McCoy’s medium (Thermo Fisher, Dreieich, Germany).
NOK pWPI and NOK HPV16 E6/E7 cells (kind gift of Ruwen
Yang and Dr. Frank Rösl, DKFZ, Heidelberg) were generated as
described17 and cultivated in Keratinocyte-SFM medium with
glutamine, supplemented with epidermal growth factor and bovine pituitary extract (Gibco). The identity
of the cells was confirmed by multiplex human cell line
authentication (SNP profiling)18 within the last 3 years. All cell
lines were tested negative for mycoplasma. HeLa mCherry H2B
and SiHa mCherry H2B (kind gift of Drs. Joschka Willemsen
and Marco Binder, DKFZ, Heidelberg) were generated from the
HeLa and SiHa cells referenced above. Experiments under hypox-
ic conditions (1% O2, 5% CO2) were performed in a hypoxic
chamber (InviO2 400 physiological oxygen workstation,
Ruskinn Technology Ltd, United Kingdom) after preincubation
of the cells for 24 hr under hypoxia. For treatment, the follow-
ing agents were used at the concentrations specified in the text:
CPX as olamine salt, deferoxamine (DFO), ferric ammonium
citrate (FAC), FeSO4, ZnSO4, ZnCl2 (Sigma-Aldrich) and
rapamycin (AdipoGen Life Sciences, San Diego, CA).

For 3D cell culture, spheroids were generated by cultivating 5,000
HeLa or 4,000 SiHa cells in 200 μl cell culture medium, supplemented
with methylcellulose, in 96-well low attachment U-bottom plates for
suspension cells (GreinerBio-One, Frickenhausen, Germany). Spher-
oids formed after 2–3 days. For the treatment of spheroids, 100 μl of
medium was removed and 100 μl medium with drugs at twice the final
concentration was added.

Protein and RNA analyses
Immunoblot analyses were performed as previously
described.19 The following primary antibodies were used:
mouse anti-HV16 E6 (Arbor Vita Corporation Sunnyvale, CA) AVC #843,19 mouse anti-HV16 E7 (NM2, kind gift of
Dr. Martin Müller, DKFZ, Heidelberg, Germany),19 mouse
anti-HV18 E6 (Arbor Vita Corporation Sunnyvale, CA) AVC #399,19 chicken anti-HV18 E7 (E7C),19 mouse anti-
Ferritin H1 #376594 (Santa Cruz Biotechnology), rabbit anti-
p21 #397 (Santa Cruz Biotechnology), mouse anti-p53 (DO-1)
#126 (Santa Cruz Biotechnology), rabbit anti-phospho (Ser15)
p53 #9284 (Cell Signaling, Boston, MA), rabbit anti-α-4E-BP1
#9451 (Cell Signaling), rabbit anti-S6K (p70S6K) #9202 (Cell
Signaling), rabbit anti-HV6 E7 (E7C),19 mouse anti-
BIM #2933 (Cell Signaling), mouse anti-β-Actin #A2228
(Sigma-Aldrich) and mouse anti-Vinculin #73614 (Santa
Cruz Biotechnology). The following secondary antibodies were
used: α-chicken IgG-HRP #2428 (Santa Cruz Biotechnology),
α-mouse IgG-HRP #2005 (Santa Cruz Biotechnology),
α-rabbit IgG-HRP (Santa Cruz Biotechnology). Enhanced
chemiluminescence using the ECL™ Prime Western Blotting
Detection Reagent (GE Healthcare, United Kingdom) was

What’s new?
Even though oncogenic human papillomaviruses (HPVs) are major human carcinogens, and metabolic alterations are a hallmark of
cancer cells, studies on the potential crosstalk between viral oncogenes and the host cell metabolism in HPV-transformed cells
remain sparse. Here, the authors show that iron chelators, including the topical antifungal agent ciclopirox, efficiently block the
expression of the viral E6/E7 oncogenes. Phenotypically, ciclopirox induces mTOR-independent senescence and, following prolonged
application, apoptosis. The findings reveal pronounced anti-viral and anti-proliferative activities of ciclopirox in both normoxic and
hypoxic HPV-positive cancer cells, highlighting the potential of ciclopirox as a novel treatment option for HPV-positive (pre)neoplasias.

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Figure 1. Legend on next page.
CPX in HPV-positive cancer cells

applied for visualization through the Fusion SL Detection System (Vilber Lourmat, Germany).

For RNA detection by quantitative real-time PCR (qRT-PCR), the ProtoScript® II First Strand cDNA Synthesis Kit (NEB, USA) was used according to the manufacturer’s protocol. cDNA was synthesized using 500 ng RNA and oligo dT primers. The samples were incubated at 42°C for 1 hr, heated up to 80°C for 5 min and then cooled down to 4°C. After the addition of 40 μl water, 2 μl of cDNA, 10 μl SYBR Green PCR master mix (Applied Biosystems, Foster City, CA), 0.4 μl of 5 pM forward and reverse primer (final concentration 0.1 pM each) and 7.2 μl water, the probes were analyzed with a 7300 Real-Time PCR System. The following primer combinations were used: HPVan E6/E7 forward: 5′-CAATGTTCCAGGACCC ACTGCG-3′, HPVan E6/E7 reverse: 5′-CTCACGTCCGCTGATA CTGTGTG-3′, HPVan E6/E7 forward: 5′-ATGCTGGAGCTA TAAGCCAC-3′, HPVan E6/E7 reverse: 5′-AGTGTCGTCTGC TGAATTC-3′, β-actin forward: 5′-GGACCTTGGACAGA GTGGC-3′, β-actin reverse: 5′-GCCAGGTATCTTCTGCA TC-3′, transferrin receptor 1 forward: 5′-TGCTGGAGACATT GGATCGG-3′, transferrin receptor 1 reverse: 5′-TATAACACA GTGGGCTGGCA-3′, p53 forward: 5′-CTGAGGTGGCTGCT TGACTGT-3′, p53 reverse: 5′-CAAAGCTTCCGCTCCCA GT-3′, 18S rRNA forward: 5′-CATGGGCTTCTTATGGTG T-3′ and 18S rRNA reverse: 5′-ATGCCAGATCTCGTTCCG TT-3′. The HPVan and HPVan E6/E7 primers recognize all three transcript classes coding for HPVan and HPVan E6/E7 in SiHa and HeLa cells, respectively. Relative quantification was performed using the comparative Ct (2ΔΔCt) method. Ct values for E6/E7 or TFR1 measurements were normalized to the reference β-actin or 18S rRNA. For statistical analysis of fold change, values were transformed logarithmically.

Colony formation, senescence and apoptosis assays

For colony formation assays (CFAs), cells were treated, then replated and cultivated in drug-free medium for the time periods indicated in the text, fixed and stained with formaldehyde-crystal violet as described before. For senescence detection, cells were treated, then replated and cultivated in drug-free medium, for the time periods specified in the text. Cells were stained for SA-β-gal activity as described and visualized by the EVOStvel Core Cell Imaging System (Thermo Fisher) with 20x magnification.

For the detection of apoptosis, TUNEL (Terminal deoxyribonucleotidyltransferase-mediated UTP end labeling) assays were performed as detailed before using the in situ cell death detection kit (Roche Molecular Biochemicals, Indianapolis, IN). Total DNA was stained with 4’,6-diamidino-2-phenylindole (DAPI, Roche Molecular Biochemicals). Apoptotic DNA strand breaks and total DNA were visualized by transmission epifluorescence microscopy. To quantify TUNEL assays, five images per coverslips were taken and the percentage of TUNEL positive cells was determined using an ImageJ Macro (written by Damir Krunic, Light Microscopy Core Facility, DKFZ). For Annexin V and PI staining, the Annexin V Apoptosis Detection Kit I for flow cytometry (BD Pharmingen, San Jose, CA) was used, following the protocol of the supplier. Cells were investigated by using the BD LSRFortessa™ cell analyzer (BD, Germany) and the BD FACS Diva Software version v8.0.1. Analyses and image generation were done with FlowJo version 10. Caspase-3/7 activation was detected by Live Cell Imaging (see below). Cleaved PARP was analyzed by immunoblot. CFAs, senescence and apoptosis assays were independently performed at least thrice with consistent results.

γ-H2AX staining

Cells were grown on coverslips, fixed, permeabilized and stained with an mouse anti-γ-H2AX antibody (P-Ser139 H2AX, Millipore, Germany), and subsequently with antimouse Cy3 (donkey α-mouse, Cy3-conjugate, Pierce antibodies) and DAPI. DNA double-strand breaks and total DNA were visualized using a Cell Observer microscope (Zeiss, Jena, Germany).

Cell cycle analyses

For cell cycle analyses, cells were harvested and processed essentially as previously described. Cell cycle distribution was analyzed by flow cytometry using the BD LSRFortessa™ cell analyzer (BD, Germany) and the BD FACS Diva Software version v8.0.1. Image generation was done with FlowJo version 10. The Dean-Jett-Fox model was used for quantification of the cells in the different cell cycle phases.

Figure 1. Iron chelators repress HPV16 and HPV18 E6/E7 oncogene expression. (a) Immunoblot analyses of E6, E7 and Ferritin expression in HeLa (left panel) and SiHa (right panel) cells treated with 100 μM DFO for the indicated time periods. -, untreated cells. β-Actin, loading control. (b) qRT-PCR analyses determining relative mRNA expression levels of HPVan E6/E7 and TFR1 in HeLa (left panels) and HPVan E6/E7 and TFR1 in SiHa (right panels) cells treated with 100 μM DFO for the indicated time periods. Expression levels were normalized to untreated control cells for each time point and are depicted as log2 fold change. Error bars indicate standard deviations (n = 4). *p < 0.05, **p < 0.01, ***p < 0.001. (c) Immunoblot analyses of E6, E7 and Ferritin expression in HeLa (left panel) and SiHa (right panel) cells after treatment with increasing doses (1–20 μM) of CPX for 12, 24 or 48 hr. β-Actin, loading control. (d) Relative mRNA expression levels of TFR1 and HPVan E6/E7 (HeLa, left panels) or HPVan E6/E7 (SiHa, central panels) cells treated with 10 μM CPX for the indicated time periods. One representative experiment is shown. Expression levels were normalized to control (EIOH)-treated cells for each time point and are depicted as log2 fold change. For the 48 hr time point, the log2 fold change was calculated from three to five independent experiments treating HeLa or SiHa cells with 10 μM CPX. E6/E7 (upper right panel) and TFR1 mRNA (lower right panel) levels are indicated relative to the solvent (EIOH) control. Standard deviations are indicated. *p < 0.01, **p < 0.001. (e) Immunoblot analyses of HPV18 E7 and Ferritin levels in HeLa cells after 48 hr treatment with 10 μM CPX. When indicated, a twofold mol excess of iron (FeSO4, FAC) or zinc (ZnSO4, ZnCl2) was added to CPX before the treatment of the cells. EIOH, solvent control. Vinculin, loading control.
Figure 2. Legend on next page.
Live cell imaging
For real-time analysis of cellular proliferation in 2D cell culture, 3,000 cells per well were seeded in 96-well plates and analyzed with the IncuCyte® S3 live-cell imaging system (Essen BioScience, Hertfordshire, UK). mCherry H2B-labeled HeLa and SiHa cells were treated with different concentrations of CPX, as specified in the text, and labeled nuclei were counted. Four images per well were acquired every 4 hr at a magnification of 10× over a period of 5 days. Analysis was performed with the IncuCyte® S3 2018A software. For the analysis of cell proliferation in 3D cell culture, HeLa and SiHa spheroids were treated with different concentrations of CPX, as specified in the text, for up to 7 days. The size of the spheroids was continuously determined with the IncuCyte® S3 spheroid module, measuring the spheroid area every 4 hr.

For detection of Caspase-3/7 activation, cells were seeded and incubated in a 96-well plate for 24 hr (HeLa and HeLa "p53 null") or for 48 hr (HCT116 and HCT116 p53+/−) and then cultivated in fresh medium containing 5 μM IncuCyte® Caspase-3/7 Green Reagent for Apoptosis (Essen BioScience, Ann Arbor, MI). This reagent couples a recognition motif for Caspase-3/7 to a DNA intercalating dye. When the nonfluorescent substrate is cleaved by activated Caspase-3 or Caspase-7, it releases the dye which then binds to DNA and leads to the emission of green fluorescence by apoptotic cells. Images were taken every 3 hr with the IncuCyte® S3 live-cell imaging system. The IncuCyte® S3 2018A software was used to analyze the number of green objects normalized to cell confluence.

Statistical analyses
SigmaPlot version 12.5 (Systat Software Inc., San Jose, CA) was used for statistical tests. For comparison of relative mRNA levels upon CPX or DFO treatment, a one-sample t-test was performed with the test mean set to zero. Shapiro–Wilk was used for normality statistic and the alpha value was set to 0.05. For the comparison of relative mRNA levels and decrease of Ferritin protein expression, respectively, upon iron depletion, statistical analysis of three independent experiments is depicted on the right. Standard deviations are indicated. **p < 0.01 (**p < 0.001) were considered statistically significant.

Data availability
The data and other items supporting the results of the study will be made available upon reasonable request.

Figure 2. CPX inhibits the proliferation of HPV-positive cancer cells. (a) Live-cell imaging (IncuCyte® system) determining the cell counts of HeLa mCherry H2B (left panel) and SiHa mCherry H2B (right panel) cells in 2D cell culture, treated with increasing doses (0.5–10 μM) of CPX for the indicated time periods. ETOH, solvent control. (b) Upper panels: Live cell imaging determining the size of HeLa (left) and SiHa (right) spheroids treated with increasing doses (2–10 μM) of CPX. Lower panels: Representative brightfield images of HeLa (left) and SiHa (right) spheroids after treatment with 10 μM CPX for 7 days. Scale bar: 500 μm. ETOH, solvent control. (c) Cell cycle analyses of HeLa cells upon treatment with solvent control (ETOH) or 10 μM CPX for the indicated time periods. The fractions of cells in G0/G1, S and G2/M phases, respectively, are indicated. (d) EdU incorporation in HeLa cells upon treatment with solvent control (ETOH) or 10 μM CPX for 2 days. One representative experiment is shown, statistical analysis of three independent experiments is depicted on the right. Standard deviations are indicated. **p < 0.01. (e) HeLa and SiHa cells were treated with 10 μM CPX for 2 days. After treatment, cells were cultivated in CPX-free medium for 4 days and subsequently analyzed by SA-β-gal assay (scale bar: 200 μm). ETOH, solvent control. (f) Concomitant CFAs, fixed 11 days after release. The experimental setup for senescence assays and CFAs is depicted in the scheme below. [Color figure can be viewed at wileyonlinelibrary.com]

Results
Iron chelators repress HPV E6/E7 oncogene expression
To analyze the effects of iron chelators on HPV-positive cells, expression of the viral E6 and E7 oncogenes were determined on mRNA and protein level upon treatment with the iron chelator deferoxamine (DFO; Figs. 1a and 1b). As indirect measure of intracellular iron levels, expression of the H-subunit of the Ferritin protein (Fig. 1a) and the transferrin receptor 1 (TFR1) mRNA (Fig. 1b) were assessed, which are expected to decrease or increase, respectively, upon iron depletions.23 As shown in Fig. 1a, DFO strongly decreases HPV E6 and E7 protein expression in a time-dependent manner in both HPV18-positive HeLa and HPV16-positive SiHa cells. Efficient E6/E7 repression by DFO is also detectable at the mRNA level as shown by quantitative real-time (qRT)-PCR analyses (Fig. 1b).

Concordant results were obtained upon treatment of HPV-positive cancer cells with the structurally unrelated iron chelator CPX, a drug used in the clinic as a topical antifungal agent.12,13,24 CPX also efficiently represses HPV16 and HPV18 E6 and E7 protein expression after 24–48 hr treatment in a dose-dependent manner (Fig. 1c). This effect is linked to a strong downregulation of E6/E7 transcript levels after 48 hr of CPX treatment (Fig. 1d). HeLa cells reproducibly showed a transient increase of E6/E7 transcript levels at 24 hr (Fig. 1d) which, however, was not linked to a detectable increase in E6/E7 protein levels (Fig. 1c). The increase of TFR1 mRNA levels and decrease of Ferritin protein levels, as indicators for iron deprivation, precede E6/E7 repression (Figs. 1c and 1d). The downregulation of the HPV oncogenes is counteracted by saturating25 CPX with a twofold molar excess of two different iron donors (FeSO4 and ferric ammonium citrate or FAC) but not by providing a two-fold molar excess of zinc (ZnSO4, ZnCl2; Fig. 1e). Taken together, these findings show that endogenous HPV16 and HPV18 E6/E7 expression is highly sensitive to iron deprivation as it is efficiently inhibited by iron chelators.

CPX inhibits the proliferation of HPV-positive cancer cells and induces senescence
Next, we analyzed the phenotypic consequences of CPX treatment in HPV-positive cancer cells. CPX induces an efficient, dose-dependent growth inhibition of HeLa and SiHa cells in 2D cell culture, as assessed by live-cell imaging (Fig. 2a). Moreover,
CPX also blocks the growth of HeLa and SiHa cells in 3D (spheroid) cell culture in a dose-dependent manner (Fig. 2b). Flow cytometry analyses revealed that CPX leads to an arrest in the G1 and S phases of the cell cycle, as indicated by increased G1 populations (Fig. 2c) and inhibition of incorporation of the thymidine analog EdU into the replicating DNA during S phase (Fig. 2d).

Moreover, both HeLa and SiHa cells undergo senescence after treatment with CPX for 2 days, as indicated by positive staining for the senescence marker senescence-associated ß-galactosidase (SA-ß-gal) as well as by morphological alterations typical for senescence (enlargement and flattening of the cells, cytoplasmic extensions) (Fig. 2e). Colony formation assays (CFAs) further support this notion since substantially fewer colonies grow.
out after release from CPX treatment, in line with the induction of senescence (an irreversible growth arrest) by CPX in these cells (Fig. 2f). The induction of senescence upon CPX treatment is efficiently counteracted by saturating CPX with a two-fold molar excess of iron (FeSO₄ or FAC) but not with a two-fold molar excess of zinc (ZnSO₄ or ZnCl₂; Supporting Information Figs. S1a and S1b), indicating that—alike the inhibition of viral E6/E7 expression (Fig. 1e)—the senescence response of HPV-positive cancer cells is the result of iron deprivation by CPX. In line, treatment of HeLa cells for 2 days with a different iron chelator, DFO, also results in senescence (Supporting Information Fig. S1c, S1d).

Figure 4. CPX induces senescence in HPV-positive cancer cells under conditions of severely impaired mTOR signaling. (a) Upper part: Experimental setup. After pretreatment of the cells with 50 nM rapamycin or solvent control (DMSO) for 24 hr, 10 μM CPX or the respective solvent control (EtOH) were added. Cells were harvested at Day 2 for immunoblot analyses of P-S6K, total S6K, P-4E-BP1, HPV16/18 E7 and Ferritin protein levels in HeLa (left panel) and SiHa (right panel). Vinculin, loading control. (b) HeLa and SiHa cells released at Day 2 from CPX and Rapa treatment were examined for induction of senescence at Day 6 (see scheme in Fig. 4a). Scale bar: 200 μm. (c) CFAs of HeLa and SiHa cells treated with CPX in the presence or absence of rapamycin. Cells released at Day 2 from CPX and Rapa treatment were stained at Day 13 (see scheme in Fig. 4a). [Color figure can be viewed at wileyonlinelibrary.com]

Figure 3. CPX-induced E6/E7 repression, growth arrest and senescence induction are not linked to p53 reconstitution. (a) Immunoblot analyses of total p53, P-p53 (Ser15), p21, HPV18 E7 and Ferritin protein levels in HeLa cells treated with 10 μM CPX for 24–72 hr. EtOH, solvent control. ß-Actin, loading control. (b) Upper panel: Immunoblot analyses of HPV18 E6, HPV18 E7 and Ferritin protein levels in HeLa “p53 null” cells treated with 10 μM CPX for 12–72 hr. Lower panel: Comparative immunoblot analyses of p53 and P-p53 (Ser15) in HeLa “p53 null” (left part of the figure) and in unmodified HeLa cells (right part of the figure), treated with 10 μM CPX for the indicated time periods. EtOH, solvent control. ß-Actin, loading control. (c) Cell cycle distribution of HeLa “p53 null” cells treated with 10 μM CPX or solvent control (EtOH) for the indicated time periods. (d) EdU incorporation analysis in HeLa “p53 null” cells treated with 10 μM CPX or solvent control (EtOH) for 2 days. One exemplary experiment is shown, statistical analysis of three independent experiments are depicted to the right. Standard deviations are indicated, **p < 0.001. (e) Senescence assays in HeLa “p53 null” cells. Cells were treated with 10 μM CPX or solvent control (EtOH) for 2 days, subsequently cultivated in CPX-free medium for 6 days and subjected to SA-β-gal staining (scale bar: 200 μm). (f) Concomitant CFAs of HeLa “p53 null” cells treated with 10 μM CPX or solvent control (EtOH), stained 11 days after CPX release. [Color figure can be viewed at wileyonlinelibrary.com]
CPX represses E6/E7 and induces senescence independently of p53

Under many experimental conditions, the repression of E6/E7 leads to an increase of total p53 levels by counteracting E6-mediated p53 degradation. However, despite efficient E6/E7 repression, 2 days of CPX treatment of HeLa cells results in a reduction of total p53 protein levels (Fig. 3a) which was linked to a decrease of p53 transcript levels (Supporting Information Fig. S2). In contrast, phospho-p53 (Ser 15) protein amounts increase under CPX treatment (Fig. 3a). This phosphorylated

Figure 5. Legend on next page.
Prolonged CPX treatment induces apoptosis in HPV-positive cancer cells. CFAs in HeLa (left panel) and SiHa (right panel) cells

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response to DNA damage and CPX has recently been linked

assays of SiHa cells treated with solvent control (EtOH) or

Figure 5

CPX also efficiently blocks mTOR signaling in HPV-positive cancer cells, as indicated by the strong reduction of both phospho-S6K and phospho-4E-BP1 levels (Fig. 4a). Notably, CPX induces senescence in both HeLa and SiHa cervical cancer cells, even when pretreated with rapamycin, as indicated by positive SA-β-gal staining (Fig. 4b), and substantially reduced colony formation capacity upon release from CPX (Fig. 4c). Collectively, these data show that CPX can induce cellular senescence under conditions of severely impaired mTOR signaling.

Prolonged CPX treatment induces apoptosis in normoxic HPV-positive Cancer cells

Notably, when treatment of HPV-positive cells with CPX was prolonged from 2 to 5 days, virtually no colonies emerged after subsequent release of the cells from CPX treatment (please compare Fig. 5a with Supporting Information Fig. S1b). Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assays indicate that a growing number of cells undergo apoptosis, starting after approximately 3 days of CPX treatment (Fig. 5b) and occurring in a dose-dependent manner (Supporting Information Fig. S4). Annexin V and PI (propidium iodide) staining after 3 days CPX treatment (Fig. 5c) reveal an increase from 8.7% to 45.3% in the fraction of late apoptotic cells (Annexin V and PI positive). Apoptosis induction is also associated with the accumulation of the cleaved form of the Caspase substrate PARP (poly(ADP-ribose) polymerase) (Fig. 5d) and a steep increase in Caspase-3/7 activity, starting at approximately 3 days of CPX treatment (Fig. 5e). Collectively, these findings show that prolonged CPX treatment induces apoptosis in HPV-positive cancer cells.

Since the proapoptotic activity of the iron chelator DFO has been linked to the induction of the proapoptotic Bcl-2 protein family member Bim, we tested the effect of CPX on Bim expression. We observed that Bim expression is increased by CPX in both HeLa and SiHa cells (Supporting Information Fig. S5).

Figure 5. Prolonged CPX treatment induces apoptosis in HPV-positive cancer cells. CFAs in HeLa (left panel) and SiHa (right panel) cells exposed to 10 μM CPX or solvent control (EtOH) for 5 days. When indicated, a two-fold molar excess of iron (FeSO₄, FAC) or zinc (ZnSO₄, ZnCl₂) was added to CPX prior treatment. After CPX treatment, cells were cultivated in CPX-free medium and stained after 10 days. (b) TUNEL assays of SiHa cells treated with solvent control (EtOH) or 10 μM CPX for up to 4 days (scale bar: 50 μm). (c) HeLa cells were treated with 10 μM CPX or solvent control (EtOH) for 3 days and stained with Annexin V and PI. Annexin V⁺/PI⁺ cells represent early apoptotic cells, Annexin V⁺/PI⁻ cells represent late apoptotic cells, (d) Immunoblot analyses of PARP and cleaved PARP (cPARP) expression levels in HeLa (left panel) and SiHa (right panel) cells treated with 10 μM CPX or solvent control for the indicated time periods. (e) HeLa and HeLa “p53 null” cells treated with 10 μM CPX for 7 days in the presence of 5 μM IncuCyte® Caspase-3/7 Green Reagent for Apoptosis detection. Activated Caspase-3/7 (green count/mm²) was assessed with the IncuCyte® S3 system and normalized to the confluence (in percent) of the cells. [Color figure can be viewed at wileyonlinelibrary.com]
Figure 6. CPX induces apoptosis in hypoxic HPV-positive cells. (a) Immunoblot analyses of HPV16 E6/E7 and Ferritin expression in SiHa cells treated with solvent control (EtOH) or 10 μM CPX either for 2 days under normoxic conditions (21% O₂) or for 3 days under hypoxic conditions (1% O₂). β-Actin, loading control. (b) CFAs of HeLa or SiHa cells treated with solvent control (EtOH) or 10 μM CPX for 3 days under hypoxia. Subsequently, the cells were cultivated under normoxia in CPX-free medium and stained after 10 days. (c) Senescence assays of HeLa and SiHa cells treated with solvent control (EtOH) or 10 μM CPX for 3 days under hypoxia. Subsequently, the cells were cultivated under normoxia in CPX-free medium and subjected to SA-β-gal staining after 3 days (scale bar: 200 μm). The experimental setup for experiments under hypoxia, as depicted in subfigures (a–c), is indicated in the scheme below. (d) TUNEL assays of SiHa cells treated with solvent control (EtOH) or 10 μM CPX under hypoxic conditions for 3–6 days (scale bar: 50 μm). [Color figure can be viewed at wileyonlinelibrary.com]
To assess a possible role for p53 during CPX-induced apoptosis, we comparatively analyzed the response of HeLa “p53 null” cells. Live-cell imaging indicates that CPX treatment ultimately leads to a similar extent of Caspase-3/7 activation in HeLa and HeLa “p53 null” cells (Fig. 5e), indicating that the proapoptotic effect of CPX is not dependent on p53. Yet, p53 appears to be important for setting the time course of CPX-induced apoptosis, since Caspase-3/7 activation is accelerated in the presence of p53 (Fig. 5e). To test if this observation is a peculiarity of HeLa cells, we compared the response toward CPX of p53 wild-type HCT116 colon cancer cells and HCT116 p53−/− cells, the latter bearing deletions in both TP53 alleles.16 Both cell lines exhibited a similar extent of Caspase-3/7 activation under CPX treatment, but this process was again accelerated in the presence of functional p53 (Supporting Information Fig. S6).

Finally, we compared the apoptotic response of NOK (normal oral keratinocytes) cells which were either transduced with an HPV16 E6/E7-expressing lentiviral vector (NOK HPV16 E6/E7) or with the corresponding empty control vector (NOK pWP).17 CPX induced apoptosis in both cell populations, but more efficiently in the HPV16 E6/E7-expressing cells (Supporting Information Fig. S7). These results suggest that keratinocytes expressing the HPV oncogenes are sensitized towards CPX treatment.

### CPX treatment induces apoptosis in hypoxic HPV-positive cancer cells

The observation that CPX can induce mTOR-independent senescence raises the question whether CPX can also act pro- or antiproliferative in HPV-positive cancer cells under hypoxic conditions, where mTORC1 signaling is impaired.7 As previously reported,7 hypoxia leads to a strong repression of viral E6/E7 expression (Fig. 6a). CPX treatment of hypoxic HPV-positive cancer cells further reduces the already low residual E7 levels, whereas E6 expression is undetectable (Fig. 6a). Notably, and in contrast to the induction of senescence after 2 days of CPX treatment under normoxia (Fig. 2e, Supporting Information Fig. S1a), both HeLa and SiHa cells do not exhibit signs of increased senescence when treated with CPX for 2 days under hypoxia (Supporting Information Fig. S8). This is evidenced by comparable SA-β-gal activation rates as untreated control cells (Supporting Information Fig. S8a) as well as by similar colony formation capacities, upon release from 2 days CPX exposure and subsequent cultivation under normoxia (Supporting Information Fig. S8b).

However, when HPV-positive cancer cells were treated with CPX for 3 days (instead of 2 days) under hypoxia and subsequently released from CPX and recultivated under normoxia, colony formation was clearly diminished (please compare the CFAs after CPX treatment at 1% O2 in Fig. 6b with those in Supporting Information Fig. S8b). These findings reveal an antiproliferative effect of CPX after prolonged treatment of hypoxic HPV-positive cancer cells. Whereas hypoxic HPV-positive cancer cells exposed to 3 days CPX treatment show no increase in the number of SA-β-gal-positive cells (Fig. 6c), they exhibit enhanced apoptosis over time, as revealed by TUNEL staining (Fig. 6d). Collectively, these data indicate that hypoxic HPV-positive cancer cells are sensitive to prolonged treatment with CPX, resulting in their elimination by induction of apoptosis.

### Discussion

The present work uncovers that iron chelators, such as CPX or DFO, can efficiently block HPV E6/E7 oncogene expression, both at the RNA and protein level. Detailed analyses of the effects of CPX on the phenotype of HPV positive cancer cells show a strong repression of cellular proliferation in both 2D and 3D cell culture and induction of cellular senescence. Interestingly, CPX-induced senescence of HPV-positive cancer cells appears to be p53-independent and, furthermore, occurs under conditions of severely impaired mTOR signaling. Moreover, prolonged exposure of HPV-positive cancer cells to CPX results in induction of apoptosis in both normoxic and hypoxic HPV-positive cancer cells. Collectively, these findings indicate that HPV oncogene expression as well as the tumorigenic phenotype of HPV-positive cancer cells are highly sensitive to iron deprivation. They also uncover the potential of CPX in efficiently activating tumorsuppressive pathways, that is, senescence and/or apoptosis, in both normoxic and hypoxic HPV-positive cancer cells.

In addition to the strong downregulation of the HPV oncogenes, the antiproliferative, prosenescent and proapoptotic effects of CPX in HPV-positive cancer cells are further supported by the reported potential of CPX to interfere with cellular pathways linked to tumorigenesis. For example, CPX has been reported to block growth-promoting signaling cascades, such as Wnt39 or mTOR35,40 signaling, and to inhibit specific iron-dependent enzymes, including ribonucleotide reductase which is required for DNA synthesis.24 Moreover, CPX has been shown to inhibit the iron-dependent activity of deoxypyrusine hydroxylase (DOHH), an enzyme which is required for the synthesis of the eukaryotic translation initiation factor 5A-1 (eIF5A1).41,42 Expression of eIF5A1 is important for the proliferation of eukaryotic cells and, interestingly, has been recently reported to be activated by the HPV16 E6 protein.33 The antiproliferative potential of CPX has also been linked to its ability to induce the proteolytic degradation of the pro-proliferative Cdc25A protein.44 Further, CPX can inhibit histone methylases, thereby epigenetically affecting gene expression.35 Most of these CPX-induced effects have been attributed to its function as an iron chelator. Our results indicate that also the repression of the HPV oncogenes and induction of senescence by CPX occurs through iron deprivation, since both responses can be blocked by excess iron and are also induced by treatment with a structurally unrelated iron chelator, DFO.

Functional investigation of senescence induction by CPX revealed two interesting mechanistic features. First, CPX can induce senescence in HeLa “p53 null” cells, suggesting that p53 is not essential for this process. This is surprising, given the key
role for p53 in the induction of cellular senescence under many conditions, including in HPV-positive cancer cells in response to E6/E7 repression. However, there is also evidence for regulatory pathways able to induce senescence independently of p53. For example, different TAp63 isoforms, members of the p53 protein family, can induce senescence in a p53-null background. Notably, the TAp63β isoform can be degraded by the HPV E6 oncoprotein. It will thus be interesting to investigate in future studies the effects of CPX on the regulation and function of p63 proteins in HPV-positive cancer cells. Second, CPX induces senescence under conditions of severely impaired mTOR signaling and, by itself, is a strong inhibitor of mTOR signaling in HPV-positive cancer cells. This observation is interesting in the light of the concept that—under many circumstances—active mTOR signaling is a key requirement for senescence induction by converting a reversible growth arrest into irreversible senescence (geroconversion). Furthermore, studies indicate that active mTOR signaling is responsible for the secretion of protumorigenic components by the SASP (senescence-associated secretory phenotype) of senescent stromal fibroblasts, which is considered to be a major obstacle for the efficacy of prosenescent anticancer therapies, including prosenescence chemotherapy and radiotherapy (CT, RT). Our data indicates that CPX utilizes an mTOR-independent pathway for senescence induction, and it will be interesting to determine whether the SASP of cells after CPX-induced senescence may be less tumorigenic than the SASP induced by other prosenescent agents, including CT and RT.

Whereas exposure of HPV-positive cancer cells for 2 days to CPX triggers subsequent induction of senescence, prolonged treatment over 3 days or longer programs the cells to undergo apoptosis. The potential of CPX to induce apoptosis is supported by a previous study indicating that CPX can diminish expression of antiapoptotic proteins, such as Survivin and Bcl-XL, and can induce cleavage of the antiapoptotic Bcl-2 protein. Moreover, we found that CPX treatment induces expression of the proapoptotic Bim protein, further supporting the notion that iron chelators induce Bim expression and raising the possibility that Bim activation may be involved in CPX-induced apoptosis. Corroborating the results of the present investigation, CPX-induced apoptosis was reported to be p53-independent, although we observed an influence of p53 on the time course of Caspase-3/7 activation, which was accelerated in the presence of p53. Interestingly, CPX also induced apoptosis in hypoxic HPV-positive cancer cells which are typically more resistant to conventional anticancer therapies in the clinic, including CT and RT.

Due to its antitumorigenic potential, CPX increasingly gains attention for being repurposed as a novel anticancer agent. A clinical study applying CPX olamine in an oral formulation in patients with hematological malignancies was discontinued due to the low bioavailability upon oral administration and dose-limiting gastrointestinal toxicity. Recently, a clinical trial in bladder cancer patients was initiated, administering a phosphoryl-oxyethyl ester of CPX (fosciclopirox) by intravenous injection. The systemic application of iron chelators has been associated with severe side effects. Notably, however, and in contrast to most other clinically used iron chelators, CPX can be applied topically, and is used since decades for the treatment of fungal infections of the skin and mucosa with an excellent pharmacological safety profile. This may make CPX particularly interesting for accessing HPV-induced (pre)neoplasias, which typically affect skin and mucosa. Furthermore, the topical application route may yield high local drug concentrations and avoid side effects associated with the systemic administration of iron chelators.

The here observed profound antitumorigenic effects of CPX on the phenotype of HPV-positive cancer cells, which include the repression of the HPV oncogenes, the induction of senescence under conditions of impaired mTOR and p53 signaling, and the induction of apoptosis upon prolonged treatment of both normoxic and hypoxic cells, indicate that further preclinical and clinical exploration of the therapeutic potential of CPX against HPV-positive (pre)neoplastic lesions is warranted.

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