Differential Induction of Apoptosis and Senescence by the DNA Methyltransferase Inhibitors 5-Azacytidine and 5-Aza-2’-Deoxycytidine in Solid Tumor Cells

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Abstract

Epigenetic alterations are a hallmark of cancer that govern the silencing of genes. Up to now, 5-azacytidine (5-aza-CR, Vidaza) and 5-aza-2’-deoxycytidine (5-aza-dC, Dacogen) are the only clinically approved DNA methyltransferase inhibitors (DNMTi). Current effort tries to exploit DNMTi application beyond acute leukemia or myelodysplastic syndrome, especially to solid tumors. Although both drugs only differ by a minimal structural difference, they trigger distinct molecular mechanisms that are highly relevant for a rational choice of new combination therapies. Therefore, we investigated cell death pathways in vitro in human hepatoma, colon, renal, and lung cancer cells and in vivo in chorioallantoic membrane and xenograft models. Real-time cancer cell monitoring and cytokine profiling revealed a profoundly distinct response pattern to both drugs. 5-aza-dC induced p53-dependent tumor cell senescence and a high number of DNA double-strand breaks. In contrast, 5-aza-CR downregulated p53, induced caspase activation and apoptosis. These individual response patterns of tumor cells could be verified in vivo in chorioallantoic membrane assays and in a hepatoma xenograft model. Although 5-aza-CR and 5-aza-dC are viewed as drugs with similar therapeutic activity, they induce a diverse molecular response in tumor cells. These findings together with other reported differences enable and facilitate a rational design of new combination strategies to further exploit the epigenetic mode of action of these two drugs in different areas of clinical oncology. Mol Cancer Ther; 12(10): 2226–36. ©2013 AACR.

Introduction

The 2 DNA methyltransferase inhibitors (DNMTi), 5-azacytidine (5-aza-CR, Vidaza) and 5-aza-2’-deoxycytidine (5-aza-dC, decitabine, Dacogen) are clinically approved for the treatment of myelodysplastic syndrome and acute myelogenous leukemia (AML). While both drugs are able to induce complete responses and hematologic improvements, a prolonged overall survival could only be shown for 5-aza-CR, but not for 5-aza-dC (1, 2). However, at the moment there is no specific guideline that governs which of these two substances should be preferred in a specific clinical context.

The development of novel dosing strategies or the combination with other antitumor therapies hold promise to exploit the activities of DNMTi compounds for an improved therapeutic effect either in hematologic malignancies or even in hitherto unaddressed tumor entities, such as solid tumors. In accordance with this rationale, several clinical studies currently investigate the application of 5-aza-CR or 5-aza-dC in combination with other anticancer strategies, including chemotherapeutic or targeted agents. For a purposeful combination of DNMTi substances with other anticancer principles it is of utmost importance to exactly define the individual tumor cell response pattern to each distinct DNMTi compound. Although both DNMTi only minimally differ in their molecular structure, there are important differences in their molecular mode of action (3, 4).

As a general principle, successful antitumor strategies have to efficiently trigger death pathways. In recent years, therapy-induced senescence (TIS) has become an important issue in the field of tumor biology and cancer therapy.
It is well known that senescence as a permanent growth arrest can be induced by a variety of stimuli, ranging from oxidative stress, DNA damage, oncogenic stress, telomere shortening, or epigenetic alterations (5–7). However, the concept of TIS as a new mechanism of anticancer agents emerged by the observation that some well known drugs can potently trigger tumor cell senescence (8–10). Thus, a therapeutically induced permanent growth arrest seems to be an attractive new option that might be suitable for a broad range of tumor entities (7). However, it is currently not clear whether the success of such a concept requires a second step that subsequently eliminates the resulting senescent cancer cells.

5-aza-dC has been reported to induce senescence by upregulation of p16(INK4a) in oral squamous cell carcinoma and heptocellular carcinoma cell lines (11, 12). In contrast, comparable results for 5-aza-CR are missing. As compounds with TIS activity should ideally not be used in combination with antimetabolites that require cell division, such as antimetabolites, we set out to characterize and compare in detail the reaction pattern of both drugs, 5-aza-CR and 5-aza-dC, for different solid tumor entities. The findings of this study clearly show a substantially different response of cancer cells to both drugs. Together with already reported characteristics of 5-aza-CR and 5-aza-dC, these results should be carefully taken into account during the development of future study protocols that intend to modulate the methylome of cancer cells by DNMTi application, especially in combination with additional anticancer drugs.

Materials and Methods

Cell culture and reagents
The human cell lines HepG2, Hep3B, A-498, HCT-116, and A549 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). According to DSMZ standards PCR-based short tandem repeats analysis as cell line authentication were conducted. The HCT-116/p53−/− cells were obtained from B. Vogelstein (Johns Hopkins University, Baltimore, MD). Disruption of the p53 gene in the HCT-116/p53−/− cells was verified by Western blotting. All cell lines were stored in liquid nitrogen, passaged for less than 4 months and cultured in Dulbecco’s modified Eagle medium with 10% fetal calf serum and 1% l-glutamine. Cells were plated in 6-, 24-, or 96-well plates and treated 24 hours later with the indicated substances. Media and supplements were from Life Technologies. 5-azacytidine (5-aza-CR) and 5-aza-2’-deoxycytidine (5-aza-dC) were obtained from Sigma-Aldrich and staurosporine from Biomol.

Real-time cell analysis
HepG2 cells (1 × 10⁵ cells/well) or Hep3B cells (2.5 × 10⁵ cells/well) were seeded in 96-well plates (E-Plate 96, Roche Applied Science). Real-time dynamic cell proliferation was monitored in 30 minutes intervals for more than 106 hours using the xCELLigence RTCA SP system (Roche Applied Science) and cell index values were calculated using the RTCA Software (1.0.0.0805). All curves were normalized at the beginning of the treatment period, 10 hour after seeding, applying the RTCA Software.

Sulforhodamine B-assay
For the sulforhodamine B-assay (SRB) HepG2 cells (2 × 10⁴ cells/well) were seeded in 24-well plates and treated with the indicated concentrations of 5-aza-CR or 5-aza-dC. After 96 hours cells were fixed with 10% trichloroacetic acid for 30 minutes at 4°C. After drying wells were stained with 200 μL 0.4% SRB solution and incubated at room temperature for 10 minutes. Plates were washed with 1% acetic acid until supernatant was colorless and dried again. SRB was resuspended in 200 μL 10 mmol/L Tris base per well for 10 minutes on ice and absorption was measured at 550 nm.

Determination of cell death
Apoptosis was determined by measuring the activity of executioner caspases and by fluorescence-activated cell sorting (FACS) quantification of hypodiploid cells. For the caspase assay 1 × 10⁴ HepG2 or Hep3B cells per well were seeded in 96-well plates, treated with different concentrations of 5-aza-CR or 5-aza-dC for 36 hours or 48 hours and then subjected to the Caspase-Glo 3/7 assay (Promega) as described by the manufacturer. As a positive control for caspase activation cells were treated with 5 μmol/L staurosporine. For FACS analysis of sub2N peaks 7.5 × 10⁴ HepG2 or Hep3B cells per well were seeded in 24-well plates and treated with different concentrations of 5-aza-CR or 5-aza-dC. After 24, 48, or 72 hours cells were stained in hypotonic buffer with propidium iodide for 30 minutes and then analyzed in a flow cytometer. Hypodiploid (sub2N) cells were considered apoptotic.

Measurement of the cellular diameter
HepG2 cells (7.5 × 10⁴ cells/well) were cultured on cover slips for 24 hours in 24-well plates. Each cover slip was transferred to a separate cell-culture chamber of a PANsys 3000 system (PAN-Systech GmbH) and treated with 20 μmol/L 5-aza-CR or 5-aza-dC. The identical observation point in each chamber was monitored for a total time span of 96 hours by phase-contrast microscopy. The maximum diameter of 20 representative cells in each cell-culture chamber after 48, 72, and 96 hours incubation was measured with ImageJ digital imaging software (ImageJ; http://rsbweb.nih.gov/ij/download.html).

Senescence-associated β-galactosidase staining
HepG2, Hep3B, A-498, HCT-116, HCT-116/p53−/−, and A549 cells were plated in 6-well plates at a density of 2 × 10⁵ cells per well and treated with the respective agents. After 72 hours (HepG2 and Hep3B) or 96 hours (A-498, HCT-116, and A549), cells were washed with PBS and fixed in 2% paraformaldehyde/glutaraldehyde before the staining with X-gal solution according to the manufacturer’s instructions (Senescence Cell Histochemical
and A549 cells were plated in 6-well plates at a density containing 20 μmol/L 5-aza-CR or 5-aza-dC for 72 hours. Cells were washed in PBS and fixed in ice-cold methanol/acetone (1:1) for 20 minutes on ice. Then, cells were washed in PBS and incubated in IF-buffer (PBS, 4% bovine serum albumin, 0.05% saponin) for 1 hour with shaking. Subsequently, rabbit anti-H3K9me3 (1:500, Cell Signaling Technology) and mouse anti-α-tubulin (1:500, DM1a, Sigma-Aldrich) antibodies were applied in IF-buffer at 4°C overnight. Samples were washed twice in PBS and incubated with secondary antibodies (1:500, chicken anti-mouse Alexafluor-594 and chicken anti-rabbit Alexafluor-488, Invitrogen) in PBS for 3 hours with shaking. After washing in PBS, samples were incubated in PBS containing 4',6-diamidino-2-phenylindole (100 μg/mL, Sigma-Aldrich) for 10 minutes and finally mounted in fluorescence mounting medium (DAKO). Images were taken using a Zeiss Axiovert 200 M microscope (63× oil immersion objective) equipped with an ApoTome and AxioVision software.

Detection of DNA double-strand breaks

DNA double-strand breaks were assayed by a histone H2A.X Phosphorylation ELISA (CyclEx Co., Ltd.). To this end, HepG2 cells (3 × 10⁴ cells/well) were seeded in 96-well plates and at the following day, treated with 5, 10, 20, 50, and 100 μmol/L of 5-aza-CR or 5-aza-dC. Forty-eight hours after treatment, histone H2A.X phosphorylation was measured according to manufacturer’s protocol.

Western blotting

HepG2, Hep3B, A-498, HCT-116, HCT-116/p53⁻/⁻, and A549 cells were plated in 6-well plates at a density of 2 × 10⁵ cells per well, treated with indicated amounts of 5-aza-CR or 5-aza-dC and incubated for 24 hours. Immunoblotting was conducted with anti-p53 (1:500, Santa Cruz Biotechnology Inc.) and anti-vinculin (1:60,000, Sigma-Aldrich) antibodies.

Densitometric analysis

X-ray films of Western blots and cytokine profiling arrays were digitized with an imaging system (FujiChem 8900, Alpha Innotech). Densitometric analyses were conducted using ImageJ (version 1.6.0_14). In brief, bands were surrounded by rectangle and plotted. The background was subtracted from the obtained peak and the area under the peak was calculated.

Cytokine profiling

HepG2 cells (2 × 10⁴ cells/well) were seeded in 24-well plates and treated with 20 μmol/L 5-aza-CR or 20 μmol/L 5-aza-dC for 72 hours. Supernatant was harvested, centrifuged at 5,000 rpm for 3 minutes and transferred to a new tube. Profiling of 36 different human cytokines, chemokines, and acute phase proteins in the cell supernatants was conducted according to the manufacturer’s instructions (Human Cytokine Array Panel A, R&D Systems). Detection was conducted by the ECL Western blotting detection system on Hyperfilm-ECL (Amersham Biosciences).

Animal treatment protocol

Housing, tumor inoculation, and drug treatment were conducted in collaboration with the Institute of Experimental Oncology (Oncotest GmbH). In brief, NMRI mice received an inoculation of HepG2 hepatoma cells into the right and left flank. When palpable tumors became detectable, animals were divided randomly into 3 groups: intraperitoneal injection of vehicle only (control group; 5 mice), 5-aza-CR (0.8 mg/kg; 6 mice), or 5-aza-dC (0.8 mg/kg; 6 mice). Animals were treated once daily and sacrificed after 3 days by carbon dioxide asphyxiation. All animal experiments were carried out in agreement with German laws concerning the conduct of animal experimentation.

Histology and immunohistochemistry

Tumor specimens were removed from the xenografted mice, fixed in formalin, and embedded in paraffin. Serial sections were routinely stained with hematoxylin and eosin (H&E). After deparaffinization of the tissue sections and heat-induced antigen retrieval immunohistochemistry was conducted using an anti-p16(INK4a) antibody (E6H4, Roche MTM Laboratories) and a Ventana Benchmark XT System (Ventana Medical Systems). The histologic analysis was conducted by a pathologist in a blinded fashion.

Chorioallantoic membrane assay

The chorioallantoic membrane (CAM) assay was done as described (13). Briefly, 1 × 10⁶ HepG2 cells were implanted on fertilized chicken eggs on day 8 of incubation and treated with vehicle (control) or 5 to 10 μmol/L 5-aza-CR and 5-aza-dC, respectively. After 3 days, tumors were sampled with the surrounding CAM, fixed in 4% paraformaldehyde, embedded in paraffin, cut in 5 μm sections, and stained with H&E. The histologic analysis was conducted by a pathologist in a blinded fashion.

Statistical analysis

Statistical analyses were conducted either with an unpaired Student’s t test or a Mann–Whitney test using GraphPad Prism Version 4.00 (GraphPad Software). According to this analysis, the following 3 different P values were examined: a P value of 0.01 to 0.05 (†), a P value of 0.001 to 0.01 (‡), and a P value < 0.001 (***).
Results

**Differential effects of 5-aza-CR and 5-aza-dC on cellular morphology**

To investigate the cellular reaction patterns to 5-aza-CR and 5-aza-dC, we first used real-time measurements of the cellular impedance using the xCELLigence SP system (14, 15) over a 96-hour time period. The cellular impedance is depicted by the cell index and a reduction of the cell index indicates reduced viability or induction of apoptosis, whereas increased cell index values indicate proliferation or increased cell size (6, 15, 16). Unexpectedly, we observed differential effects of 5-aza-CR and 5-aza-dC treatment on HepG2 and Hep3B hepatoma cells for all tested concentrations (Fig. 1A). Incubation of both cell lines with 5-aza-CR at concentrations from 5 μmol/L to 50 μmol/L led to a reduction of the normalized cell index in comparison with untreated cells. In contrast, 5-aza-dC treatment increased the normalized cell index of HepG2 cells. In additionally conducted experiments, a cell index increase was even found for 1 and 0.5 μmol/L of 5-aza-dC (data not shown). Interestingly, this 5-aza-dC-mediated effect was not detectable in Hep3B cells. The most prominent difference between these 2 hepatoma cell lines is their different p53 status: HepG2 are p53 wild-type expressing cells, whereas Hep3B cells are p53-deficient (17).

![Figure 1.](image-url) Distinct effects of 5-aza-CR and 5-aza-dC on tumor cell proliferation, viability and cell size. A. real-time cell monitoring of HepG2 and Hep3B hepatoma tumor cells over 106 hours. Cells were seeded in a microplate, treated with the vehicle control or the indicated concentrations of 5-aza-CR and 5-aza-dC, respectively, after 10 hours and observed for additional 96 hours. Cellular impedance was measured continuously using the xCELLigence SP system and is depicted as cell index. Displayed are cell index values obtained every 5 hours, normalized at the time point of treatment. Treatment with Triton 0.1% X-100 was used as positive control for cell death. Shown are mean values ± SD of a representative experiment out of three independent experiments carried out in triplicate. B. sulforhodamine B assay of HepG2 cells treated with the indicated concentrations of 5-aza-CR and 5-aza-dC for 96 hours. Shown are mean values ± SD of four independent experiments, each carried out in duplicate; Mann–Whitney test; **, P < 0.01; ***, P < 0.001. C. 5-Aza-dC induces an increase of the cellular diameter. Changes in cellular morphology were investigated by measuring cellular diameter of HepG2 cells upon treatment with vehicle or 20 μmol/L of 5-aza-CR or 5-aza-dC for 48, 72, and 96 hours. The diameter of 20 cells per group was measured in a randomized fashion and calculated as mean ± SD; Mann–Whitney test; **, P < 0.01; ***, P < 0.001; ns, not significant.
To further characterize the differential results of the real-time cell monitoring for 5-aza-CR and 5-aza-dC, a sulfonhydrabine B cytotoxicity assay was conducted (Fig. 1B). Incubation of HepG2 cells with 5-aza-CR resulted in a dose-dependent decline of viability. The viability of HepG2 cells was significantly reduced by 5-aza-CR at all concentrations tested in comparison with 5-aza-dC (Fig. 1B; ***, P < 0.001). In addition, measurement of the cellular diameter was conducted after 48, 72, and 96 hours of treatment with 20 μmol/L 5-aza-CR or 5-aza-dC (Fig. 1C and Supplementary Table S1). This concentration was chosen to ensure a strong demethylating potency for both compounds. While 5-aza-CR treatment did not alter the cell size, a significant increase in the cellular diameter was observed when HepG2 cells were incubated with 5-aza-dC for 72 or 96 hours (Supplementary Table S1; ***, P < 0.001). These results comply with the findings of the real-time cell monitoring and indicate that the 5-aza-dC-mediated increase of the cell index in the p53 wild-type cell line HepG2 was based on an increment of the cell size, which is a known hallmark of cellular senescence.

5-aza-dC but not 5-aza-CR induces cellular senescence

To further investigate a potential senescence induction by the DNMTi 5-aza-dC, the expression of the senescence-associated marker β-galactosidase (SA-β-gal) was determined. HepG2 and Hep3B cells were treated for 72 hours with 20 μmol/L 5-aza-CR or 5-aza-dC. This time point was chosen due to the divergent curve progression in the real-time monitoring and the results of the cellular size measurement. Incubation with 5-aza-dC, but not 5-aza-CR, led to a significant increase (**, P < 0.001) in the number of SA-β-gal-positive HepG2 cells (Fig. 2A) displaying a characteristic blue staining (Fig. 2B).

Another marker for senescence is the accumulation of trimethylated histone H3 lysine-9 (H3K9me3), which is involved in the formation of so-called senescence-associated heterochromatin foci (SAHF; refs. 18, 19). We therefore investigated SAHF formation by immunofluorescence microscopy of H3K9me3 in HepG2 cells (Fig. 2C). In line with the previous results and unlike 5-aza-CR, treatment with 20 μmol/L 5-aza-dC for 72 hours triggered not only the typical increase of cell size, but also resulted in the characteristic accumulation of H3K9me3 in subnucleolar dots. Together, our in vitro results clearly indicate that 5-aza-dC induces cellular senescence in p53 wild-type HepG2 cells.

We further analyzed the induction of senescence in an in vivo xenotransplant model. Noteworthy, in comparison with the in vitro situation senescent cells often lack to display all classical markers of senescence in vivo (9). Especially the commonly used SA-β-gal staining has at least in some models not been a reliable marker for cellular senescence. Thus, other classic senescence markers, such as the cell-cycle inhibitor p16(INK4a) have been suggested to be applied instead (9). In our model, we therefore investigated on the one hand p16(INK4a) expression as a marker of senescence and on the other hand conducted H&E staining to compare p16(INK4a) staining with the overall cellular viability status of the tumor cells. For this purpose, nude mice harboring subcutaneously implanted HepG2 xenografts were treated intraperitoneally with either the solvent control, 5-aza-CR or 5-aza-dC. To choose a comparable experimental setup to the in vitro experiments, the in vivo grown tumors were explanted after only 72 hours of treatment. Hence, a reduced tumor volume was not expected but the p16(INK4a) expression and formation of necrosis was analyzed. In this experimental setting, only 5-aza-dC but not 5-aza-CR induced an upregulation of p16(INK4a), whereas 5-aza-CR treatment caused a more pronounced increase in necrosis in the tumor tissues (Fig. 2D). Thus, our in vivo experiments support the in vitro findings of senescence induction by 5-aza-dC.

Another feature of senescence is the induction of the senescence-associated secretory phenotype (SASP), which comprises various cytokines, growth factors, or soluble receptors that are released from senescent cells (16, 20, 21). We therefore measured the SASP components in supernatants of HepG2 cells treated for 72 hours with 20 μmol/L 5-aza-CR or 5-aza-dC. The incubation with 5-aza-dC caused increased levels of sICAM-1, interleukin (IL)-1ra, and IL-8 in the culture supernatants (Fig. 2E and Supplementary Fig. S1). Interestingly, all three molecules have previously been associated with cellular senescence (22–25). In contrast with 5-aza-dC, 5-aza-CR led to a reduced secretion of sICAM-1 and IL-1ra, which might be a result of protein synthesis inhibition by this compound. IL-8, however, was upregulated by both DNMTi (Fig. 2E). Interestingly, increased IL-8 expression has been described as a molecular marker for hepatotoxicity in HepG2 cells (26) as well as under various apoptotic conditions in other cell types (27, 28). Hence, induction of IL-8 expression by 5-aza-CR could rather be explained as a sign of cytotoxicity than as an effect of cellular senescence. Of note, both drugs did not induce an increase of IL-8 in p53-deficient Hep3B cells.

DNMTi 5-aza-CR directly activates cell death instead of cellular senescence

5-aza-CR possesses a ribonucleoside structure and can be incorporated into both RNA and DNA, whereas 5-aza-dC, based on its deoxyribonucleoside structure, can be inserted into DNA only (3, 29, 30). Because of this different chemical structure 5-aza-CR seems to be more toxic and a less potent DNMTi at comparable concentrations. To determine whether 5-aza-CR induces other cellular pathways than senescence, apoptosis was analyzed by flow cytometric measurement of cells with hypodiploid DNA (Fig. 3A and B). Incubation with 5-aza-CR resulted in a dose-dependent increase of hypodiploid HepG2 and Hep3B cells after 48 and 72 hours, whereas 5-aza-dC showed only a marginal alteration in HepG2 cells. Because of the prodrug characteristics of 5-aza-CR and to exclude a too low dosage of 5-aza-CR in comparison...
with 5-aza-dC also a concentration of 100 μmol/L was tested by FACS analysis at these two time points. Again, 5-aza-CR displayed a pronounced increase in the sub2N fraction under this dosage (Supplementary Fig. S2A and S2B). In addition, caspase-3/7 activity (being used as an indicator for apoptosis) was assessed after 5-aza-CR or 5-aza-dC treatment, including the 100 μmol/L concentration (Supplementary Fig. S2C and S2D).

Treatment with the two DNMTi for 36 and 48 hours resulted in activation of the executioner caspases upon exposure to high concentrations of 5-aza-CR but not 5-aza-dC. Thus, 5-aza-CR reduces the cell viability by caspase activation resulting in apoptosis of both p53-proficient and -deficient tumor cells.

To further investigate the effects of 5-aza-CR and 5-aza-dC on cell death induction in an in vivo model, both compounds were used in a CAM assay with HepG2 cells. Representative images of the H&E-stained sections showed a profound necrosis by 5-aza-CR treatment, whereas 5-aza-dC had no relevant effect (Fig. 3C). Thus, 5-aza-CR is able to induce caspase activation, cell death and substantial tumor necrosis in vivo. Notably, this observation is in clear contrast with the senescent phenotype induced by 5-aza-dC.
5-aza-dC induces profound DNA damage, whereas 5-aza-CR reduces cellular p53 levels

Several DNA damage pathways can induce cellular senescence. We therefore analyzed the occurrence of double-strand breaks upon 5-aza-CR or 5-aza-dC treatment by measuring the phosphorylation of H2A.X in HepG2 cells (Fig. 4A). In line with published data (3), we detected a significant increase of phosphorylated γH2A.X by 5-aza-dC in a similar concentration range that induced senescence. 5-aza-CR, in contrast, only caused marginal DNA-damaging effects (Fig. 4A; **, P < 0.01, ***, P < 0.001).

Unlike 5-aza-dC, 5-aza-CR is known to block RNA synthesis (3, 30), followed by inhibition of protein synthesis, which might especially affect short-lived proteins, such as p53 (3, 4). For this reason, the protein levels of p53 in HepG2 cells were determined by Western blotting after 5-aza-CR or 5-aza-dC treatment (Fig. 4B). Notably, p53 levels showed a dose-dependent decline, which started to occur already within 24 hours of treatment with 5-aza-CR but not 5-aza-dC (Fig. 4B). Of note, as described in the literature, no expression of p53 was detectable in Hep3B hepatoma cells (Supplementary Fig. S3). In addition,
alterations of p53 levels were investigated in other p53 wild-type harboring tumor cells, including HCT-116 colon, A498 kidney, and A549 lung carcinoma cells. Remarkably, in all tumor cell lines, a dose-dependent decline in p53 protein levels was detected upon 5-aza-CR treatment (Fig. 5A and Supplementary Fig. S4A and S4C). In contrast, 5-aza-dC rather led to an increase in p53 protein levels, which might be related to the senescence-inducing capacity of this drug. In line with p53 wild-type HepG2 cells, treatment with 20 μmol/L 5-aza-dC induced a significant increase of SA-β-gal staining in these solid tumor cells (Fig. 5B and Supplementary Fig. S4B and S4D). Most interestingly, HCT-116/p53−/− colon carcinoma cells lacking p53 displayed an altered reaction pattern. Treatment with 20 μmol/L 5-aza-CR or 5-aza-dC did not induce a significant increase of the percentage of β-galactosidase–positive tumor cells emphasizing the essential role of p53 for 5-aza-dC-mediated TIS (Fig. 5C and D).

Discussion

Both, 5-aza-CR and 5-aza-dC were originally developed as nucleoside antimetabolites for anticancer therapy (30, 31). Both drugs are analogues of cytidine and share the same basic chemical structure with only small differences in the ribose backbone. For this reason 5-aza-CR and 5-aza-dC are supposed to exhibit a similar mode of action by inhibiting cellular DNA methyltransferases (31). Both azanucleosides have been shown to be effective in the treatment of myelodysplastic syndrome and AML and are currently the only U.S. Food and Drug Administration-approved DNMTi compounds (3). In some tumor models, such as oral squamous-cell carcinoma or hepatocellular carcinoma, 5-aza-dC induces cancer cell senescence (11, 12). This has led to the hypothesis that either DNA hypomethylation or an alteration of the DNA structure might be involved in senescence induction (8, 32). Therefore, it might be reasoned that compounds with DNMTi activity generally induce tumor cell senescence by DNA demethylation. However, according to our in vitro and in vivo experiments, only 5-aza-dC induced a senescent phenotype in p53 wild-type tumor cells. In contrast, a dose-dependent reduction of viability, induction of cell death and decline of p53 protein levels were observed upon 5-aza-CR treatment in different tumor cell lines. These results further imply important differences of these two closely related and clinically applied demethylating compounds in solid tumor cells. Concerning AML as a disease in which both substances are currently in clinical use, a recent publication reported, for example that only about 30% of tumor cells with a complex karyotype are p53 wild-type (33). To our knowledge, it is not known so far, whether p53 wild-type patients with AML differentially respond to 5-aza-CR and 5-aza-dC. As a starting point from our article it would be interesting to directly compare these response patterns in different AML populations, which could possibly lead to the identification of patient subgroups that benefit most from either 5-aza-CR or 5-aza-dC.

Possible explanations for the distinct cellular response patterns of 5-aza-CR and 5-aza-dC are versatile. Concerning DNMTi activity, 5-aza-dC is regarded to be a more

Figure 4. 5-aza-dC induces DNA double-strand breaks, whereas 5-aza-CR reduces p53 protein levels. A, determination of phosphorylated H2A.X as a marker for DNA double-strand breaks in HepG2 cells after treatment with the indicated concentrations of 5-aza-CR or 5-aza-dC; Mann–Whitney test; *P < 0.05; **P < 0.01; ***P < 0.001. B, Western blot and densitometric analysis of p53 content in HepG2 cells after 24 hours of treatment with vehicle or the indication of 5-aza-CR or 5-aza-dC. Expression of the house keeping protein vinculin served as a control for equal protein loading. Relative p53 levels were determined by densitometric analysis.
potent inhibitor than its ribonucleoside analogue 5-aza-CR (3, 34). Furthermore, even at similar concentrations 5-aza-CR and 5-aza-dC induce quite different changes in gene expression patterns, as for example, shown in several comparative gene array analyses, mainly in AML cells (34–36). In light of these data, it is not surprising that both substances differ in their mode of action, including the induction of senescence in p53 wild-type solid tumor cells exclusively by 5-aza-dC. Noteworthy, an elegant recent study that investigated the transient application

![Figure 5. 5-aza-CR induces a decrease of p53 protein levels, whereas 5-aza-dC increases β-galactosidase activity p53 dependently. A and C, Western blot and densitometric analyses of p53 content in cell lysates of HCT-116 wt (wild-type) and HCT-116/p53−/− colon carcinoma cells treated with vehicle or increasing concentrations of 5-aza-CR or 5-aza-dC over a 24 hours time period. Vinculin expression served as a loading control. B and D, after 96 hours of incubation with vehicle or 20 μmol/L 5-aza-CR or 5-aza-dC, HCT-116 wt and HCT-116/p53−/− cells positive for SA-β-gal and nuclei were counted via microscopy. Bars represent mean ± SD of three independent experiments; Student t test; **, P < 0.01; ns, not significant.](image)

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of 5-aza-CR and 5-aza-dC reported that already low doses exerted distinct alterations in several signaling pathways in leukemia cell lines and primary cancer cells, respectively (37).

Another contribution to TIS induction might be the higher amount of DNA strand breaks that are triggered by 5-aza-dC compared with 5-aza-CR (38). It is well known that DNA damage signaling pathways are designated activators of cellular senescence (7) and that p53 plays an essential role in DNA damage-induced senescence in tumor cells (5, 6). Our results are in line with these findings, showing senescence induction in functional p53-harboring HepG2, but not in p53-deficient Hep3B cells. Interestingly, due to a 5-aza-CR-mediated inhibition of protein synthesis a dose-dependent decline of p53 levels was detected not only in hepatoma-derived, but also in p53 wild-type cells of other tumor entities including colon, renal, and lung cancer. Notably, in contrast with HCT-116 colon carcinoma cells, the p53-deficient derivative HCT-116/p53−/− cell line did not develop TIS under 5-aza-dC treatment. The observed p53 dependency is in line with reports showing that restoration of p53 induces cellular senescence and upregulation of SASP components in liver carcinomas (39, 40). Interestingly, loss of p53, as by 5-aza-CR treatment, generally facilitates tumor growth and an aggressive malignant phenotype (39).

In summary, our data illustrate that the 2 closely related DNMTi nucleosides analogues 5-aza-CR and 5-aza-dC induce a substantially different reaction pattern in tumor cells. This observation is supported by our previous data (4) and other reports showing distinct effects of both compounds on antiproliferative activity, cytotoxicity, gene demethylation, transcription, natural killer (NK) cell activity, or DNA repair (34, 36, 41–49). A comprehensive overview of these differences is summarized in Table 1. Thus, although 5-aza-CR and 5-aza-dC are often viewed as mechanistically similar, our results clearly emphasize specific differences between both drugs, which have to be considered for the further development of these compounds, especially in combination approaches with other anticancer strategies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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