Sensitization by 5-Azacytidine toward Death Receptor-Induced Hepatic Apoptosis

Timo Weiland, Markus Weiller, Gerald Künstle, and Albrecht Wendel

Biochemical Pharmacology, Faculty of Biology, University of Konstanz, Konstanz, Germany

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ABSTRACT

5-Azacytidine (5-aza-CR) is a DNA-hypomethylating antineoplastic agent used because of its inhibitory activity on DNA methyltransferases. Today, it is approved as an epigenetically active drug therapy for treatment of myelodysplastic disorders, with a contraindication as to pre-existing liver diseases. Because the mechanism of its hepatotoxicity is still unknown, we investigated the pharmacodynamic properties of 5-aza-CR with regard to death receptor/ligand-induced apoptosis and the mode of execution of cell death. In a time- and concentration-dependent manner, primary murine, human hepatocytes and HepG2 cells exposed to 5-aza-CR became highly sensitive toward cell death induced by CD95L, tumor necrosis factor (TNF)-related apoptosis-inducing ligand, or TNF. Cell death was characterized as apoptotic by membrane blebbing, chromatin condensation, and exposure of phosphatidylserine on the outer membrane. Neither 5-aza-2′-deoxycytidine nor the common DNA methyltransferase inhibitors S-(5′-adenosyl)-l-homocysteine or RG 108 showed any significant effects under these conditions. Despite the complete protection of HepG2 by high concentrations of the pan-caspase inhibitor N-benzoyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (z-VAD-fmk), effector caspase-3/7 activity was completely abolished at approximately a 20-fold lower concentration of z-VAD-fmk. Under these conditions, the serine protease inhibitors Nα,toly-l-phenylalanine chloromethyl ketone, N,p-tosyl-l-lysine chloromethyl ketone, and 4-(2-aminoethyl)-benzenesulfonfluoride, respectively, conferred protection against death receptor ligands. We conclude that this caspase-independent apoptosis is executed by a yet-unidentified serine protease.

Among agents used in the therapy of malignant diseases, the so-called antimetabolites are common. These substances interfere within mechanisms of cellular homeostasis, including cell division. Because of their chemical relationship to mostly purine/pyrimidine bases, these agents, when incorporated into DNA/RNA, disturb the stability of the genome and, hence, also protein expression, with the consequence that cell division of rapidly dividing cells is preferentially affected. From this, tumor growth is retarded, and accelerated cell demise may eventually stop malignancy (Cole et al., 2005). In fact, the cytidine analog 5-azacytidine and its derivative, 5-aza-2-deoxycytidine, was first synthesized as a potential chemotherapeutic long before its inhibitory activity on DNA methyltransferases was recognized (Hrodek and Vesely, 1971).

The methylation of cytosine within promoter regions of genes is a common epigenetic mechanism to control genome regions, e.g., to remain in a transcriptionally inactive state over a long line of successor cells or for cellular differentiation and tissue formation in development. A misguided methylation pattern may lead, however, to genetic instability and undesirable repression of tumor suppressor genes. In contrast to DNA defects induced by mutation that alter the primary sequence, epigenetic changes are in principle reversible. With the increasing awareness of the importance of a dysfunctional epigenetic regulation of gene expression as a driving mechanism of carcinogenesis, the interest in molecules interfering with DNA methylation grew (Yoo and Jones, 2006).

5-aza-CR and its analog, 5-aza-2′-deoxycytidine (5-aza-dCR), are actively incorporated into nucleic acids, where they bind irreversibly to DNA methyltransferases, thereby depleting the active DNA-methylating capacity of cells. As a result, these compounds have the potential to reverse aberrant gene expression.

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ABBREVIATIONS: 5-aza-CR, 5-azacytidine; 5-aza-dCR, 5-aza-2′-deoxycytidine; TLCK, N,p-tosyl-l-lysine chloromethyl ketone; TPCK, Nα-tosyl-l-phenylalanine chloromethyl ketone; AEBSF, 4-(2-aminoethyl)-benzenesulfon fluoride; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; DR, death receptor; DTT, dithiothreitol; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; AcT, actinomycin D; CHX, cycloheximide; z-VAD-fmk, N-benzoyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone; NF, nuclear factor; RG 108, 2,1,3-dioxo-1,3-dihydro-2H-isoinold-2-y1-3-(1H-indol-3-yl)propionic acid.
silencing because of methylation errors. Because DNA methylation is a long-term gene-silencing regulator, such drugs act only on newly synthesized DNA and therefore potentially in rapidly dividing target cells by reverting a misguided silencing of tumor suppressor genes, which thus restores normal physiological growth control. Today, 5-aza-CR is the first DNA-hypomethylating agent approved for treatment of myelodysplastic syndromes and represents a promising drug class for epigenetic cancer therapy (Kaminskas et al., 2005).

This study was conducted to elaborate a more detailed understanding of sensitization by methyltransferase inhibitors of hepatocytes toward endogenous death receptor ligands and the mechanism of the proteolytic execution of cell death in our model system. The aim of any therapeutic intervention strategy to overcome malignant cell growth is a maximal impact on tumor cells with preferably limited detrimental effects on normal tissue. Therefore, it is desirable to selectively induce apoptotic cell death, with the advantage that a gentle cell demise occurring without signs of inflammation or necrotic damage to surrounding tissues is initiated. Most drugs lead to apoptotic events because of damaging of DNA or other critical molecules or eventually by triggering cell death receptors. The activation of the caspase family of proteases as initiators and effectors is regarded as the central element within apoptotic cell death (Fulda and Debatin, 2006b).

As a drawback of such a strategy, it turned out that many tumor cells lose their responsiveness to apoptotic triggers during the process of transformation or during the course of chemotherapy, i.e., development of chemoresistance. Tumor cells may evade apoptosis by ineffective caspase activation, defective signal transduction, or overexpressed endogenous caspase inhibition mechanisms (Schimmer et al., 2001; Philchenkov et al., 2004). However, an entirely complete resistance of tumor cells to programmed cell death is very unlikely to occur because alternative pathways of executing apoptosis may come into function. For instance, many studies have demonstrated that noncaspase proteases also play an important role in apoptosis induced by chemotherapeutic agents and death receptor agonists utilizing redundant death signaling pathways, which may bypass defective caspase-based routes (Johnson, 2000; Bröker et al., 2005). Along this line, evidence accumulates that especially serine proteases might play a basic role in transduction and execution of at least apoptotic-like cell death (O’Connell and Stenson-Cox, 2007). The notion that serine protease may play an fundamental role in programmed cell death is especially substantiated by the observation that particular apoptotic events can be prevented by broad-range inhibitors of serine proteases such as TLCK, TPCP, or AEBSP (Egger et al., 2003; Thorburn et al., 2003; Liu et al., 2004). This redundancy may hold important implications for the development of novel types of chemotherapeutics that address caspase-independent cell death mechanisms, in which signaling and effector components remain functional even after prolonged drug exposure.

**Cell Culture Experiments.** Primary human hepatocytes were isolated from pathological inconspicuous specimens obtained from patients undergoing elective surgery of hepatic tumors. The cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum and maintained in a humidified incubator at 5% CO2/95% air at 37°C in 24-well plates. Isolation and culturing of primary hepatocytes from 8-week-old mice was performed by the two-step collagenase perfusion method (Leist et al., 1984). HepG2 (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 containing 10% fetal calf serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml) in a humidified incubator at 5% CO2/95% air. Cells were harvested and seeded out on either 96-well (cytotoxicity/activ- ity of caspases) or six-well plates (morphological analysis) in a density of 2.5 × 104 and 2 × 105 cells/well, respectively. Hepatocytes were exposed to increasing concentrations of 5-aza-CR (Cihák, 1974) or 5-aza-dCR (Winkley and Robins, 1970) 180 min before treatment with 100 ng/ml TNF-α, 10% CD95L, and 100 ng/ml TRAIL (Biomol Research Laboratories, Plymouth Meeting, PA). 5-Aza-CR and 5-aza-dCR were obtained from Sigma Chemie (Deisenhofen, Germany). Cytokines were diluted in saline supplemented with 0.1% human serum albumin. Inhibitors were predissolved in dimethyl sulfoxide and diluted to working concentrations with saline. Serine protease inhibitors were purchased from Sigma Chemie. Inhibitors were generally added 30 min before challenge with DR agonists. Final concentrations of dimethyl sulfoxide never exceeded 1%, and corresponding solvent controls were run.

**Caspase Activity Assay.** Activity in cell lysates was assayed by cleavage of the synthetic substrate DEVD in a concentration of 50 μM in caspase assay buffer (50 mM HEPEs, 50 mM NaCl, 10 mM EDTA, 10 mM DTT, 0.1% CHAPS, 5% glycerol, pH 7.2). Release of free amido trifluoromethyl coumarin was monitored in intervals of 5 min for 30 min at 37°C in a Wallac Victor2 multilabel counter (PerkinElmer Wallac, Turku, Finland). One unit of specific activity was calculated as micromoles of substrate cleavage, as assessed by release of free amido trifluoromethyl coumarin per milligram of protein per minute (picomoles per milligram per minute).

**Cytotoxicity Assays.** Cytotoxicity was measured by the reduction of the tetrazolium dye Alamar Blue (BioSource International, Camarillo, CA) by viable cells. Vehicle-treated cells were used to set the basal level of cytotoxicity (i.e., 0% cytotoxicity), cells lysed with 0.1% Triton X-100 were used to set its maximal level (i.e., 100% cytotoxicity). This method depends on a constant number of plated cells in every well for a given experiment, a basic requirement, which is not given primary human hepatocytes obtained from clinical material. Hence, to cope with the disadvantage of differing cell numbers per well, cytotoxicity was assessed by measurement of lactate dehydrogenase in culture supernatants (S) and in the remaining cell monolayer (C) after lysis with 0.1% Triton X-100 using the LDH Cytotoxicity Detection Kit (Roche Diagnostics, Mannheim, Germany). The percentage of lactate dehydrogenase release was calculated from the ratio of S/(S + C).

**SDS-Polyacrylamide Gel Electrophoresis/Western Blot.** Cultured cells were lysed with lysis buffer (250 mM sucrose, 50 mM Tris-HCl, 5 mM imidazole, 2.5 mM EDTA, 2.5 mM DTT, 0.1% CHAPS, pH 7.40), and protein concentration was measured using the Bradford protein assay (Bio-Rad, Hercules, CA). In brief, an aliquot of each sample equivalent to 30 μg of protein was boiled after addition of the appropriate amount of 5% sample buffer (5 mM EDTA, 162 mM DTT, 5% SDS, 50% glycerol, 0.5% bromphenol blue, 188 mM Tris, pH 8.8). The samples were separated on 12% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred onto nitrocellulose filters using the Bio-Rad electrotransfer system (Bio-Rad). Equal transfer was verified by Ponceau S staining of the membranes. Antibody-antigen antibody complexes were visualized with hors eradish peroxidase-coupled secondary antibody (goat anti-mouse and goat anti-rabbit; Dianova, Hamburg, Germany) and a custom-made enhanced chemiluminescence detection system (2.5 mM luminol, 0.4 mM para-coumaric acid, 10 mM...
Microscopic Determination of Apoptotic Markers. Cellular morphology and nuclear condensation in HepG2 cells were visualized by fluorescence microscopy using a fluorescein isothiocyanate filter (450 nm) after staining for 5 min at 37°C with 1 μg/ml Hoechst 33342 (Invitrogen, Carlsbad, CA). Phosphatidylserine on the outer membrane was stained with 10 μg/ml Merocyanine 540 (BIOMOL Research Laboratories). Cells were washed twice with phosphate-buffered saline before analysis with fluorescence microscopy using a rhodamine filter (590 nm).

Statistics. All data are given as means ± S.D. Statistical analysis was done by one-way analysis of variance followed by Dunnett’s multiple comparison test. All statistics were calculated using the program GraphPad Prism 4.01 (GraphPad Software Inc., San Diego, CA), and a p value < 0.05 was considered as being significant.

Results

Concentration-Dependent Induction of Cell Death by Death Receptor Agonists after Sensitization by 5-Azacytidine. In initial experiments, the capability of 5-aza-CR to cause cell death in primary human hepatocytes and in murine hepatocytes alone or in combination with death receptor ligands was tested. In concentrations up to 100 μM, 5-aza-CR as such showed no significant cytotoxicity in human and in murine hepatocytes. In contrast, in the presence of per se nontoxic concentrations of the death receptor ligands TNF-α, CD95L, or TRAIL, respectively, a concentration-dependent increase of cell death of up to 60% was found in primary human hepatocytes (Fig. 1A). Similar results were obtained with primary murine hepatocytes, wherein the treatment of cells with increasing concentrations of 5-aza-CR combined with TNF-α or CD95L, an enhanced cell death took place (Fig. 1B). The data show that 5-aza-CR sensitizes primary hepatocytes toward death receptor ligand-induced cell death. For a more detailed characterization of this type of cell death, the human hepatoma-derived cell line HepG2 was utilized as a model of liver (patho)physiology because it still expresses many hepatic functions, including induction of death receptor apoptosis (Müller et al., 1997).

In contrast to primary cells, 5-aza-CR sensitized HepG2 in a selective way toward death receptor ligands. Although CD95L and TRAIL-triggered apoptosis were increased in the presence of 5-aza-CR, no sensitization of TNF-α-induced cell death was found. At higher concentration ranges between 50 and 100 μM, 5-aza-CR exerted toxic effects per se on HepG2 (Fig. 2A). Similar findings were obtained in murine AML-12 cells; the pretreatment with 5-aza-CR was sensitized only toward CD95L-triggered cell death (data not shown).

Alterations of Expression Levels of Apoptosis-Modulating Proteins by 5-Azacytidine. To narrow down the site of action within the apoptotic pathways, where 5-azacytidine might influence the susceptibility of cells to DR-induced apoptosis, the capability of 5-aza-DR to modulate apoptosis-related proteins was determined. Treatment with 5-azacytidine increased p53 and Bax levels in a time- and concentration-dependent manner, as detectable on Western blot (Fig. 3, A and B). Furthermore, there seemed to be a defined progression of events because the p53 level preceded the alterations in Bax expression (Fig. 3C). The modulation of p53 occurred already after approximately 1 h, followed by increased Bax expression after 3 to 9 h. It is notable that sensitization toward cytotoxicity was most pronounced when the cells had been pretreated 3 h before addition of the stimuli (data not shown). Neither the known proapoptotic proteins FADD, Bid, and the caspases 3, 8, 9, nor the antiapoptotic proteins Bcl-2, XIAP, and cFLIP showed any alterations under influence of 5-aza-CR (data not shown). Furthermore, the nonsensitizing DNA methyltransferase inhibitor 5-aza-dCR was not able to interfere with any of the above-mentioned proteins, including p53 and Bax (data not shown).

Sensitization by Global Inhibition of Protein Translation? There are good reasons to believe that a nucleoside analog, when incorporated into RNA, would interfere with RNA synthesis and protein processing, eventually ending up in global inhibition of translation. This raises the question whether or not the mechanism of sensitization toward death receptor ligands is comparable with translational inhibition by CHX. In our system, both the common transcriptional inhibitor/sensitizer ActD and the translational inhibitor/sensitizer CHX showed no selective sensitization toward CD95L and TRAIL as such as ActD and CHX sensitize also toward TNF-α (data not shown). Thus, on the molecular level, 5-aza-CR and CHX lead to a divergent pattern of alteration of protein expression levels. In contrast to 5-aza-CR, CHX diminished the level of p53 over a period of 12 h (Fig. 3D).
Induction of Effector Caspase Activity in Primary Murine Hepatocytes and HepG2. The concentration dependence of cytotoxicity induced via the death receptor agonists CD95L and TNF-α in primary murine hepatocytes in the presence of 5-aza-CR correlated very well with the activity of the effector caspase-3/7 (Fig. 4, A and B). In the presence of 5-aza-CR in combination with CD95L and TRAIL, respectively, substantial activation of effector caspases was found in HepG2 cells (Fig. 4, C and D). The strong correlation between effector caspase activity and cell death confirms that activation of the caspase cascade seems to be a causal event for subsequent cell death. This interpretation is further substantiated by the kinetics after death receptor trimerization in HepG2 after treatment with 5-aza-CR/CD95L or 5-aza-CR/TRAIL. Even though the activation was more pronounced in case of TRAIL, the DEVD cleavage activity peaked around 6 to 8 h followed by cell death reaching a plateau phase after approximately 21 h (Fig. 5, A and B). In case of activation of the TNF-α pathway; however, neither caspase activity nor cell death could be seen (data not shown).

Morphology of Cell Death Shows Common Criteria of Apoptosis. To identify the nature of the observed cell death after sensitization with 5-aza-CR, common hallmarks of apoptosis were determined, i.e., condensation of chromatin within apoptotic nuclei and the presentation of phosphatidylserine on the outer membrane of apoptotically dying cells were examined. These typical features of apoptosis were detectable in primary murine hepatocytes (data not shown) and in HepG2 cells treated with 5-aza-CR/CD95L (Fig. 6).

Caspase Inhibition by z-VAD-fmk Confers Protection to Primary Murine Hepatocytes but Fails to Prevent Death Receptor Agonist-Induced Cell Death in HepG2 Cells within Caspase-Inhibiting Concentration Ranges. As expected, primary murine hepatocyte cell death induced by 5-aza-CR/CD95L treatment was abolished by increasing concentrations of the known pan-caspase inhibitor z-VAD-fmk (Künstle et al., 1997). The IC_{50} values for caspase activity and protection from cell death were similar, i.e., 20 and 23 nM (Fig. 7A). Inhibition of the caspase cascade in HepG2 cells treated with a combination of 5-aza-CR and...
CD95L, failed to afford protection (Fig. 7B). It is striking that z-VAD-fmk achieved protection against cell death only in very high concentrations up to 50 μM/H9262M, far beyond the one needed for blunting caspase activity, as indicated by the arrow in Fig. 6B. The corresponding IC_{50} values of z-VAD-fmk for half-maximal inhibition of caspase activity and cytotoxicity were 78 and 1200 nM, respectively, and 77 nM for 5-aza-CR/CD95L and 1500 nM for 5-aza-CR/TRAIL. These divergent ratios demonstrate that approximately 16 to 20-fold higher concentrations of z-VAD-fmk were necessary for half-maximal inhibition of cytotoxicity compared with half-maximal inhibition of caspase activity. HepG2 cells treated with 5-aza-CR in combination with 1.5 μM z-VAD-fmk, a concentration that completely inhibited caspase activity, failed to protect against cytotoxicity. Despite caspase arrest by z-VAD-fmk, the dying cells displayed features common to a typical apoptotic morphology, including exposure of phosphatidylserine accompanied with chromatin condensation; no signs for necrotic cell death were found (Fig. 8). In addition, we found a low-molecular weight DNA ladder (126 bp and multiples) also under caspase arrest (data not shown).

**Contribution of Serine Protease Activity under Caspase Arrest.** Along the lines of previous work using the classical “ActD/CHX/death receptor ligand” model, in our model HepG2 sensitized by 5-aza-CR toward death receptor/ligand-induced apoptosis, different inhibitors were screened targeting putative noncaspase effector proteases. Among these, only the combination of the serine protease inhibitors TPCK, TLCK, and AEBSF with 1.5 μM z-VAD-fmk conferred protection of HepG2 cells against 5-aza-CR/CD95L (Fig. 9). Neither of the serine protease inhibitors alone had any protective effect. In the presence of the serine protease inhibitors TPCK and AEBSF, a decrease, or at least a delay, of caspase activity measured after a fixed time point of 6 h after stimulation was observed, whereas in the presence of z-VAD-fmk, no caspase activity at all was detectable (data not shown). The inhibition by at least AEBSF can be characterized as specific for serine proteases because the inactive analog AEBSA was not effective at any concentration used (data not shown).

**Discussion**

Inhibitors of DNA methyltransferases represent a new promising class of drugs, opening new perspectives for the
treatment of myelodysplastic syndrome. The current strategies of treatment tend rather to supportive care and/or stem cell transplantation because of a lack of an effective and selective chemotherapy. The first compound of this kind, which had reached Food and Drug Administration approval, is 5-azaeytidine. Only recently, the inhibitory action of 5-aza-CR and its analog, 5-aza-dCR, on DNA methyltransferase became obvious as the link between defects in DNA methylation and cancer arose. Many subsequent studies showed that inhibition of DNA methyltransferase activity can lead to antineoplastic effects. Especially 5-aza-dCR is able to overcome chemoresistance against TRAIL-induced apoptosis in various tumor cell lines like glioblastoma, neuroblastoma, and nonsmall lung cancer cell lines (Eramo et al., 2005; Fulda and Debatin, 2006a).

Currently, a popular approach to tumor therapy addresses means to use epigenetic modulation to hit tumor cells including hepatomas. This in vitro study was designed to comprise malignantly transformed liver cells and primary mouse and human hepatocytes for examining the acute effects of 5-aza-CR on death receptor-induced apoptosis. Our study reveals that under special conditions, 5-aza-CR might impair cellular integrity of untransformed hepatocytes by sensitizing them toward endogenous death-inducing cytokines. The pharmacological significance of such a sensitization by azacytidine places a strong caveat as to its clinical use, even beyond to pre-existing liver diseases.

The other DNA methyltransferase inhibitor screened in this study showed no sensitizational effects in the chosen setup of coincubation with cytokines in either hepatocyte type used. Thus, it appears that the sensitization by 5-aza-CR on hepatocytes seems to be an exclusive property of this compound, unrelated to general DNA methyltransferase-inhibitory activity. It has to be taken into account, however, that 5-aza-CR and 5-aza-dCR could lead to an unspecific inhibition of transcription and translation. Because of its ribonucleoside structure, 5-aza-CR is incorporated into DNA as RNA and thus might affect protein translation (Santini et al., 2001). Because 5-aza-CR exhibits enhanced cytotoxicity during the S phase, it should have greater impact on DNA rather than on RNA replication (Momparler et al., 1984). In our system, ActD and CHX used for comparison were capable of sensitizing HepG2 toward all three cytokines used. Therefore, the lack of sensitization effect of 5-aza-CR

![Fig. 6. Morphological analysis of 5-aza-CR/CD95L-induced apoptosis. Morphology (top), chromatin condensation (middle), and phosphatidylserine exposure (bottom) of HepG2 cells treated with saline (left column), 75 μM 5-aza-CR (second column), 10% (v/v) CD95L (third column), or the combination of both compounds (right column). Six hours after challenge with CD95L, chromatin condensation and exposure of phosphatidylserine were visualized by staining with Hoechst-33258 (1 μg/ml) and merocyanin-540 (5 μg/ml). Representative pictures of two independent experiments are shown in 400× magnification. White bar, 25 μm.](image-url)

![Fig. 7. Concentration-dependent inhibition of caspase activity and cell death by z-VAD-fmk. Effect of z-VAD-fmk on caspase activity (open symbols, dotted line) and cytotoxicity (closed symbols) in primary murine hepatocytes (A) and HepG2 (B), respectively, treated with: diamonds, 75 μM 5-aza-CR; and triangles, 100 ng/ml TRAIL, 75 μM 5-aza-CR, and 10% (v/v) CD95L. Caspase activity and cytotoxicity were determined after 8 and 21 h, respectively. Cytotoxicity was measured by Alamar Blue. Representative graphs derived from two independent experiments are shown. Data represent mean ± S.D.](image-url)
experiments are shown. Data represent mean by Alamar Blue. Representative graphs derived from two independent
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alone or in combination with serine protease inhibitor 25
Noxa, and stabilized p53, depends on the stimulus or cause of
caspase-8 upon receptor trimerization), Bim, Bad, PUMA,
BH3-only molecules like tBid (as generated by activation of
per se, it needs a direct activator. The switch, in this case
way, which amplifies death signals. Because Bax is not active
old of susceptibility to apoptosis via the mitochondria path-
tration of Bax to Bcl2 constitutes a rheostat, setting the thresh-
discussion of Bax in treated cells by a yet-unknown
model” (Boucher-Hayes et al., 2005). 5-Azacytidine is likely
tication by 5-azacytidine according to the “switched rheostat
for the sensitization of cell death in hepatocytes treated with the death receptor agonist.
One explanation for the selective sensitization might be that there are major differences in the activation of the transcription factor NFκB between primary hepatocytes and neoplastically transformed liver cells. The stimulation of TNF receptor 1 by binding of TNF-α leads to pleiotropic effects within the cells, which allows for the induction of apoptosis when NFκB, a repressor of apoptosis, fails to be activated (Beg and Baltimore, 1996; Micheau and Tschopp, 2003). In contrast to primary hepatocytes, 5-aza-CR might either stimulate NFκB, directly or indirectly, or fail to block its action in HepG2. However, a stimulated, proliferative pathway activated by 5-aza-CR would also negatively affect apoptosis triggered by CD95L and TRAIL. Thus, the direct activation of NFκB by 5-aza-CR seems to be little likely to explain our findings.
Another possible explanation for selective sensitization might come from studies by Friesen et al. (1996) and Müller et al. (1998), who have shown that apoptosis induced in tumor cells involves the CD95 system. Treatment of hepatoma cell lines with chemotherapeutic agents causes up-regulation of the CD95 ligand and receptor, leading to cell death. Up-regulation of the CD95 system in hepatocellular carcinomas was controlled by activation of p53 after DNA damage and, therefore, sensitized cells to apoptosis (Chatterjee et al., 2001). The TRAIL receptors DR4 and DR5 have also been demonstrated to be induced by DNA-damaging agents in a p53-dependent fashion (Guan et al., 2001; Takimoto and El-Deiry, 2001). Therefore, a possible up-regulation of p53 by 5-azacytidine as a DNA-targeting agent could contribute to selective sensitization of HepG2 cells toward CD95L and TRAIL triggered apoptosis.
Selective, tightly controlled proteolysis is a defining characteristic of apoptosis, resulting in the ordered disassembly of the cell, confining this mode of death from uncontrolled necrosis (Kerr et al., 1972). Cellular demise with typical apoptotic morphology is induced by caspase-mediated cleavage of specific substrates like lamins, cytoskeletal proteins,
inhibitor of caspase activated DNase, or p21 kinase 2; hence, caspases occupy an outstanding role within apoptotic signaling and execution. However, it was recognized that a variety of noncaspase proteases can cleave at least some of the typical caspase substrates, thus mimicking or taking over the role of noncaspase proteases can cleave at least some of the typical caspase substrates, thus mimicking or taking over the role of caspases (Leist and Jäättelä, 2001) followed by apoptosis or at least a regulated apoptosis-like cell death. Over recent years, a number of publications described the contribution of serine proteases to apoptotic signaling and execution of apoptosis (Johnson, 2000; O’Connell and Stenson-Cox, 2007). Serine proteases are implicated since several years into possible functions in the cellular apoptosis machinery. However, the relationship and possible interdependence between serine proteases and apoptotic, caspase-based signaling still remains unclear because signaling pathways vary strongly between different cell types and stimuli used. The site of action of serine proteases may be allocated upstream or downstream of caspases or even attributed to completely caspase-independent signaling pathways. In theory, the contribution of noncaspase proteases may be executed either in concert with caspases or completely independent of them (Kidd et al., 2000; Bröker et al., 2005). Our findings that inhibition of caspases by z-VAD-fmk failed to abrogate cytotoxicity in human primary hepatocytes and in the human hepatoma cell line HepG2 strongly suggest that caspases are activated in but are dispensable for the death executioner agonist-induced, apoptosis-like cell death in this model. It is notable that the cytoprotection in HepG2 cells sensitized by 5-aza-CR by the serine protease inhibitors TPCK and TPCK could only be observable in the presence of caspase-inhibitory concentrations of z-VAD-fmk. These results confirm data by Dünzl et al. (2007) in HepG2 cells, sensitized with ActD/CHX, and extend them to 5-aza-CR sensitization with the serine protease inhibitor AEBSF. These data support the emerging paradigm change, i.e., that caspase activation is not necessarily a one-way end route of apoptosis (Bröker et al., 2005). Our findings rather favor the view that serine-based proteases may take over as a backup death pathway after cysteine protease pathways had been disengaged. It seems likely that HepG2 cells possess a redundant serine protease-based apoptotic pathway, which becomes active in the 5-azaCR/CD95L system. In conclusion, we believe that uncovering backup death pathways that can take over after blocking caspases offers a new understanding of executioner proteases in apoptosis.

These findings may also have some tangible clinical implications. The therapeutic success of traditional cancer drugs is limited by two major obstacles, namely adverse drug reactions of healthy tissues and organs and chemoresistance of tumor cells, e.g., when key components of the apoptotic machinery become dysfunctional.

Under distinct immunological conditions, a patient undergoing 5-aza-CR-including therapy may react extremely sensitively to systemic circulating cytokines such as CD95L and TNF-α. By extrapolation from in vitro to in vivo, hepatotoxic side effects observed in clinical studies (Kaminskas et al., 2005) could be explained by this sensitization against cytokines that might be released from endogenous sources during hepatic diseases (e.g., Wilson’s disease, alcohol-induced hepatitis, hepatitis B, hepatic tumors) and thus potentiate their pathophysiological effects.

A second consequence of our study to be considered is that caspase-independent apoptosis and other forms of cell death are targets to be addressed for cancer therapy (Brown and Wilson, 2003; Brown and Attardi, 2005). Efforts to concentrate on developing agents aiming to kill tumor cells apoptotically by bypassing their survival adaptations or by restoring the defects in key components of their cell death machinery seem promising new strategies derived from the elucidation of novel signaling pathways leading to apoptosis.

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Address correspondence to: Albrecht Wendel, Faculty of Biology, University 
of Konstanz, M667, D-78457 Konstanz, Germany. E-mail: albrecht. 
wendel@uni-konstanz.de