# Different DNA lesions trigger distinct cell death responses in HCT116 colon carcinoma cells

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## Abstract

**The pleiotrophic cellular response to DNA damage includes activation of cell cycle checkpoints, induction of DNA repair pathways, and initiation of programmed cell death among others. The fate of cells with damaged DNA depends on the coordination of these different responses. The clinical efficacy of genotoxic therapies is influenced by cell fate and thus by how the DNA damage response is coordinated. While a great deal has been learned about how different DNA lesions activate distinct cell cycle checkpoints and DNA repair pathways, less is known about whether the type of DNA lesion influences the qualitative and quantitative nature of the cell death response. To address this question, HCT116 colon carcinoma cells have been treated with equally cytotoxic doses of the antitumor DNA alkylating agents adozelesin or bizelesin or the DNA strand scission agent C-1027. The relative contribution of cell cycle arrest and cell death to measured cytotoxicity varied among the three drugs. Apoptotic cell death accounts for most C-1027 cytotoxicity while cell cycle arrest and cell death both contribute to the cytotoxicity of the alkylating agents. Each of the drugs induces a distinct but overlapping pattern of caspase activation. In addition, the cell death response to these drugs is differentially dependent on p53 and p21. These observations suggest that the type of DNA lesion influences not only the relative extent of apoptotic cell death at a given cytotoxic dose but also the qualitative nature of that response. [Mol Cancer Ther 2004;3(5):613–9]**

## Introduction

DNA damage elicits a multifaceted cellular response that includes activation of DNA repair pathways, cell cycle checkpoints, and programmed cell death among others. Several distinct cell fates can result from coordination of these responses. The damaged DNA can be repaired and cell cycle checkpoints released, the cell can remain in a state of extended cell cycle arrest, or the cell may undergo programmed cell death. Programmed cell death may occur by different molecular mechanisms. These distinct fates are expected to influence the clinical utility of genotoxic therapies for cancer. While a great body of literature has established that different types of DNA lesions induce distinct cell cycle checkpoints and repair pathways (1–3), less is known about whether the type of DNA lesion influences the nature of the cell death response. This gap in our knowledge limits our ability to predict the outcomes and optimize the effectiveness of genotoxic cancer therapies.

Adozelesin and bizelesin are members of the cyclopropylpyrroloindole (CPI) family of highly toxic, antitumor alkylating agents (4, 5). They bind the minor grove of DNA at A/T-rich regions and alkylate the N3 adenines at the 3′ end of the DNA binding site (6–8). Adozelesin is a monofunctional CPI while bizelesin is a CPI dimer that can alkylate adenines on one or both DNA strands possibly forming double-strand DNA cross-links (9). While both adozelesin and bizelesin create similar DNA lesions and have similar DNA sequence preferences (8), the ability of bizelesin to form cross-links may influence how the cell recognizes and responds to this drug.

C-1027 is a member of the enediyne family of radiomimetic drugs that interact with DNA and induce single-strand breaks, double-strand breaks, or abasic sites by free radical attack of deoxyribose moieties (10, 11). C-1027 is a highly cytotoxic, antitumor agent that prefers to induce double-strand lesions at the sequence GTTA/T/ATA2A3 (damage positions numbered; Refs. 10, 11). Unlike radiation, macromolecular damage induced by C-1027 is highly specific for DNA.

Adozelesin, bizelesin, and C-1027 are all highly cytotoxic, the damage they induce is highly specific for DNA, and the type of DNA lesions they produce are limited and highly specific. Hence, these drugs are ideal for comparing the effects of different DNA lesions on cell death responses without the confounding effects of damage to other cellular macromolecules or additional types of DNA lesions. Here, we test whether DNA lesions caused by these three drugs trigger qualitatively or quantitatively distinct cell death responses in mismatch repair-deficient HCT116 colon carcinoma cells.

## Materials and Methods

### Reagents

Adozelesin, bizelesin, and C-1027 were generously provided by Dr. Terry Beerman (Roswell Park Cancer Institute, Buffalo, NY) and resuspended in DMSO. 2,3-Bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay reagents were from Roche Diagnostics (Mannheim, Germany). Propidium iodide was purchased

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from Sigma Chemical Co. (St. Louis, MO) McCoy's 5A was from Mediatech Cellgro (Herndon, VA). Fetal bovine serum was purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA). Apoptosis detection kit (Annexin V staining) was purchased from R&D Systems, Inc. (Minneapolis, MN). Caspase fluorometric peptide substrates were from BioVision (Palo Alto, CA). Antibodies against cyclin B1, GADD45 (Santa Cruz Biotechnology, Santa Cruz, CA), $p21^{WAF1}$ (Pharmingen, San Diego, CA), and p53 (Oncogene Research Products, Darmstadt, Germany) were provided by the indicated commercial suppliers.

### Cell Culture

The HCT116 cell line and its p53-null or p21-null derivatives were originally provided to Dr. Terry Beerman by Prof. B. Vogelstein (Johns Hopkins University, Baltimore, MD). The cells were cultured in McCoy's 5A containing 10% fetal bovine serum in an atmosphere of 5% $CO_2$. To maintain a constant drug to DNA mass ratio in all the experiments, the volume of media used to culture the cells was 1 ml/$1.6 \times 10^4$ cells. Cells were plated 1 day prior to drug treatment. Cells were incubated with drug containing media for 4 h, the media was removed, the cells washed twice with PBS, and incubation continued in fresh media without drug.

### Assays

For cytotoxicity assays, cells in 96-well plates were seeded and drug treated as above. Two days later, the relative number of viable cells was assayed by XTT assay as recommended by the manufacturer. The cells were incubated with assay reagent for 4 h prior to determination of $A_{490}$ in a plate reading spectrophotometer (Bio-Tek Instruments, Winooski, VT). The $A_{490}$ values were normalized to the vehicle control (DMSO). Each data point was calculated as the mean of three independent experiments, each done in triplicate. For each drug, the normalized $A_{490}$ value was plotted against the log drug concentration. The least squares method was used to derive the equation describing the line best fitting the data. This equation was used to calculate the $IC_{50}$ and $IC_{90}$.

Apoptotic cell death was assayed 20 h after drug treatment by measuring surface exposure of phosphatidylserine; exposed phosphatidylserine was stained by FITC-conjugated Annexin V according to manufacturer's recommendations. The cells were stained simultaneously with propidium iodide to exclude nonviable cells. The percentage of Annexin V-positive cells was counted on a FACScan flow cytometer using Lysis II software (Becton Dickinson, Franklin Lakes, NJ). At least 5000 gated cells were counted for each sample.

Caspase activity was measured using fluorometric peptide substrates as recommended by the manufacturer. Initially, cleavage of nine peptide substrates was assayed using extracts prepared from drug-treated cells 0, 2, 4, 12, or 20 h after $IC_{50}$ or $IC_{90}$ drug treatment. Based on the results of this initial screen, subsequent assays were performed 2 h postdrug treatment to identify the initial caspases activated. The various peptide substrates used in the assays contained preferred cleavage sites for caspases 1–10. Because preferred peptide substrates for caspases 2, 3/7, and 9 were cleaved most extensively in the 2 h postdrug treatment extracts, we focused the presented analysis on these substrates. Peptide cleavage assays were performed at 37°C for 2 h and the samples were read in a Bio-Tek fluorometer.

Cell cycle distribution was measured by propidium iodide staining and flow cytometry as described (12). At least 10,000 gated cells were counted for each sample. The histogram data were curve fit and the percentage of cells in each phase of the cell cycle was estimated using ModFit software. Cells were treated with $IC_{50}$ or $IC_{90}$ of drug for 4 h and incubated in drug-free media for an additional 44 h before fixation and analysis.

### Western Blot Analysis

Cells were collected 20 h after drug treatment and extracted with Lysis 250 buffer [50 mM Tris-HCl (pH 7.4), 250 mM NaCl, 5 mM EDTA, 0.1% NP40, 50 mM NaF], protein concentration was determined using the Bradford assay, and equal mass of total cell protein resolved by SDS-PAGE. SDS-PAGE gels were blotted onto polyvinylidene difluoride membrane and the membranes were blocked and stained with primary antibodies using standard methods. Blots were developed by chemiluminescence using horseradish peroxidase-conjugated secondary antibodies according to manufacturer's recommendations (Amersham, San Francisco, CA).

## Results

### Drug Effects on Proliferation of HCT116 Cells

Accumulation of viable cells, as measured by XTT assay, was used to assess the cytotoxicity of adozelesin, bizelesin, or C-1027 relative to vehicle-treated controls. This short-term assay was used to measure the immediate cell cycle and cell death effects of each drug. Cells were treated with each drug for 4 h and incubation continued for 2 days in drug-free media; 4 h treatment is sufficient for CPI DNA alkylation (13) and strand scission by C-1027 (14). All of the drugs significantly reduced cell proliferation at low nanomolar concentrations (Fig. 1). Adozelesin was the most potent compound, inhibiting cell proliferation to 50% of untreated controls ($IC_{50}$) at 0.27 nM. The $IC_{50}$ for adozelesin determined here is consistent with previous published data in several human tumor cell lines, including HCT116 (15–17). However, the $IC_{50}$ values of bizelesin and C-1027 measured here (1 and 2 nM, respectively) were higher than the low picomolar $IC_{50}$ values measured for each of these compounds previously. This difference is likely due to the different assays used to assess cytotoxicity; previous studies used long-term clonogenic survival to assess cytotoxicity. Long-term clonogenicity assays are likely to be influenced by mechanisms of cytotoxicity, such as cellular senescence and delayed cell death, which are not detected in the short-term assay described here.

The *p53* gene is an important regulator of cell cycle, repair, and cell death responses to DNA damage (18–20). The p21 protein can be induced by both p53-dependent and p53-independent mechanisms; it is critically important for

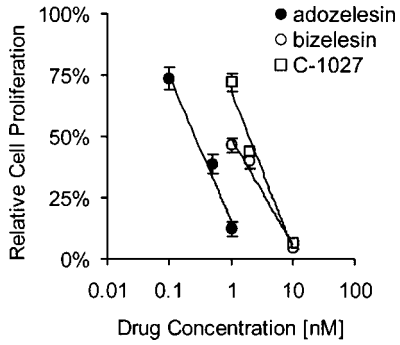


**Figure 1.** Drug cytotoxicity in HCT116 colon carcinoma cells. Cells at constant number/volume culture media were treated for 4 h with each of the drugs at the indicated concentrations. Cells were washed and fed with drug-free media and incubation continued for 2 days. The number of viable cells relative to vehicle-treated control was measured using XTT assay. *Filled circles,* adozelesin; *open circles,* bizelesin; *open squares,* C-1027. Data are expressed relative to the vehicle-treated control. *Points,* mean of three independent experiments done in triplicate; *bars,* SD. The trend line was calculated using least squares method.

enforcing cell cycle checkpoints and may also negatively regulate apoptotic cell death (21, 22). To test the possibility that the cytotoxicity of these drugs may be differentially dependent on p53 or p21, we compared drug effects on two cell lines isogenic with HCT116 but for mutational inactivation of p53 or p21. Cell proliferation of vehicle-treated cultures for wild-type, p53-null, or p21-null lines were not significantly different. Loss of p53 had little effect on the $IC_{50}$ or $IC_{90}$ values for each drug relative to wild-type cells (Table 1). Loss of p21, however, sensitized HCT116 cells to the three drugs as indicated by a 5-fold reduction in the $IC_{50}$ of adozelesin and a 2-fold decrease in the $IC_{50}$ of bizelesin or C-1027.

That loss of p53 did not significantly affect the toxicity of HCT116 cells to these DNA damaging drugs was surprising given its important role in the regulation of the DNA damage response. One possible explanation was that the DNA lesions produced by these drugs at the doses used did not efficiently trigger activation of p53. We examined the levels of p53, p21, and GADD45 protein in HCT116 either treated or untreated with each drug at $IC_{50}$. Each of the drugs was able to induce an increase in p53 levels indicative of p53 activation (Fig. 2A). Levels of *p21* and *GADD45*, two genes known to be activated by p53 (23, 24), were also increased on treatment by each of the drugs. In p53-null HCT116 cells, however, the levels of p21 and GADD45 protein were unchanged after drug treatment (Fig. 2B). These data suggest that each of the drugs could activate a p53 response at $IC_{50}$.

**Table 1. Drug concentrations (nM) required for 50% and 90% relative growth inhibition ($IC_{50}$ and $IC_{90}$)**

| Cell lines | Adozelesin (nM) | | | Bizelesin (nM) | | | C-1027 (nM) | | |
|---|---|---|---|---|---|---|---|---|---|
| | $IC_{50}$ | $IC_{90}$ | $R^2$ | $IC_{50}$ | $IC_{90}$ | $R^2$ | $IC_{50}$ | $IC_{90}$ | $R^2$ |
| HCT116WT | 0.27 | 1.2 | 0.98 | 0.99 | 7.9 | 0.98 | 2.0 | 8.1 | 0.98 |
| HCT116p53−/− | 0.32 | 0.92 | 1.0 | 1.6 | 7.6 | 0.90 | 2.3 | 7.6 | 0.95 |
| HCT116p21−/− | 0.05 | 0.75 | 1.0 | 0.48 | 5.3 | 0.90 | 0.88 | 7.0 | 0.95 |

Note: The indicated cell lines were treated with the drugs at various concentrations for 4 h and incubated for an additional 2 days in drug-free media. The number of viable cells relative to vehicle-treated control cultures was determined by XTT assay. The results of three independent experiments were averaged and the data were graphed *versus* the log drug concentration. The least squares method was used to derive the equation describing the line best fitting the data. The equation was used to estimate the $IC_{50}$ and $IC_{90}$ concentrations. The coefficient of determination ($R^2$) is given for each data series.

### Drug Effects on Cell Cycle Responses in HCT116 Cells

Cell cycle checkpoint activation and cell death are likely to account for most of the short-term effects of drug treatment observed. We examined the cell cycle distribution of cells treated with $IC_{50}$ doses of adozelesin, bizelesin, and C-1027 to test if the relative contribution of cell cycle arrest to the observed cytotoxicity varied depending on the type of DNA lesion. Both adozelesin and bizelesin induced a decrease in the percentage of $G_1$-phase cells ($P = 0.001$ and 0.0003, respectively, for paired Student's $t$ test) and an increase in the percentage of cells in the $G_2$-M phases of

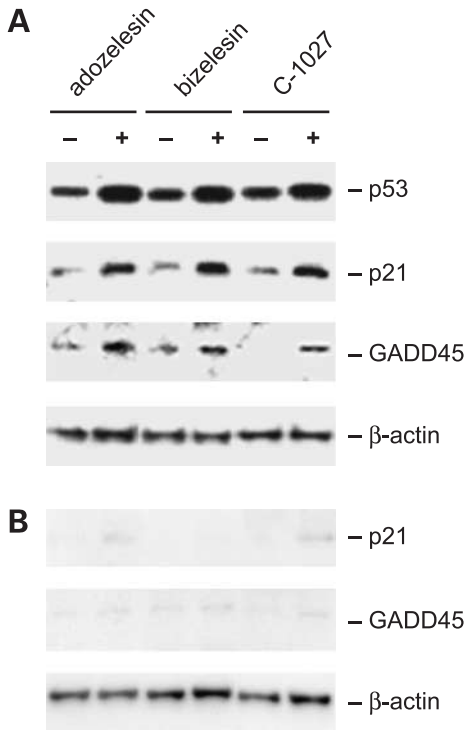


**Figure 2.** Drug-induced activation of the p53 pathway. **A,** HCT116 cells were treated with $IC_{50}$ of each drug or vehicle control as indicated. Cells were washed and fed with drug-free media and incubation continued for 20 h. Cells were extracted and equivalent mass of total cell protein was resolved by SDS-PAGE. The gels were Western blotted and blots were stained with antibodies directed against the indicated proteins. β-actin serves as a loading control. Data are representative of two independent experiments. **B,** the p53-null HCT116 cell line was treated and analyzed as in **A**.

the cell cycle ($P$ = 0.0006 and 0.01) consistent with activation of a $G_2$-M checkpoint (Fig. 3). Adozelesin-induced and bizelesin-induced activation of a $G_2$-M checkpoint was at least partially dependent on p21 because neither drug induced a statistically significant increase in the percentage $G_2$-M phase cells in the absence of p21. C-1027 did not trigger any change in the relative percentage of cells in the $G_1$, S, or $G_2$-M phases of the cell cycle consistent with a lack of cell cycle checkpoint activation.

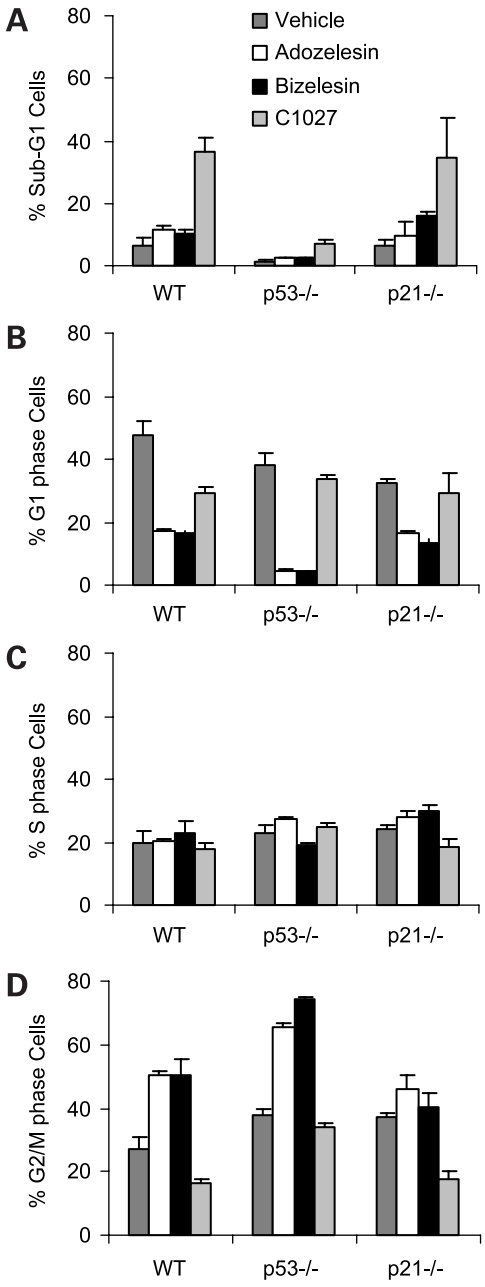


**Figure 3.** Drug-induced changes in cell cycle phase distribution. **A,** wild-type (*WT*), p53−/−, or p21−/− HCT116 cells were treated with the indicated drug at $IC_{50}$ for 4 h followed by a 20 h incubation in drug-free media. The cell cycle distribution of the cells was determined by propidium iodide staining and flow cytometry. The percentage of cells in the sub-$G_1$ phase of the cell cycle is shown. *Columns,* mean of three independent experiments; *bars,* SD. **B,** as in **A**, the percentage of $G_1$-phase cells. **C,** as in in **A**, the percentage of S-phase cells. **D,** as in **A**, the percentage of $G_2$-M-phase cells.

To confirm the $G_2$-M cell cycle arrest induced by adozelesin and bizelesin, we have examined the levels of cyclin B1 protein in drug-treated cells. Total cyclin B1 levels oscillate during the cell cycle and peak at $G_2$-M where cyclin B/Cdk1 activity is required for mitosis. Relative to untreated control cells, cells treated with either $IC_{50}$ adozelesin or bizelesin but not C-1027 show increased levels of cyclin B1 protein (Fig. 4A). No relative change in cyclin B1 expression was observed in drug-treated HCT116 cells lacking p21 in keeping with the observed lack of $G_2$-M cell cycle arrest (Fig. 4B).

**Drug Effects on Cell Death Responses in HCT116 Cells**

Cells treated with C-1027 at $IC_{50}$ do not exhibit pronounced changes in cell cycle distribution, suggesting that cell cycle checkpoint activation does not make a major contribution to C-1027 toxicity. C-1027 does induce an increase ($P$ = 0.003) in the percentage of sub-$G_1$ cells (Fig. 3). Cells with sub-$G_1$ DNA content are typically undergoing genome fragmentation, a process consistent with apoptotic cell death. C-1027-induced genome fragmentation does not absolutely require p53 or p21 because a statistically significant increase in the percentage of sub-$G_1$ cells relative to vehicle control is observed in p53-null or p21-null HCT116 cells ($P$ = 0.008 and 0.08, respectively). However, the absence of p53 clearly decreases the percentage of sub-$G_1$ cells, indicating that p53 status does influence C-1027-induced cell death.

C-1027 is capable of fragmenting DNA directly. However, the doses used are not expected to produce a sufficient number of DNA lesions to account for the extensive DNA fragmentation observed. To confirm that the DNA fragmentation observed is due to apoptotic cell death, Annexin V staining has been used as a marker of apoptosis in drug-treated cells (25). As predicted, there is a significant increase ($P$ = 0.0006) in the percentage of Annexin V-positive cells on treatment with C-1027 relative to vehicle-treated control (Fig. 5). Annexin V staining is also induced on $IC_{50}$ treatment of cells with adozelesin and bizelesin ($P$ = 0.03 and 0.05, respectively), although the extent of increase in Annexin V-positive cells is less.

Consistent with the DNA fragmentation data above, C-1027 induced significant increases in the percentage of Annexin V staining cells relative to vehicle control ($P$ = 0.003) even in the absence of p53, although the total number of Annexin V staining cells was reduced. Loss of p21 sensitized both vehicle-treated and drug-treated cells to apoptotic cell death, although bizelesin and C-1027 still induced a significant increase in the percentage of Annexin V staining cells ($P$ = 0.02 and 0.002, respectively).

The above experiments indicated that there were quantitative differences in the relative extent of apoptotic cell death induced by the drugs at equally cytotoxic doses. To test whether there were qualitative differences in the cell

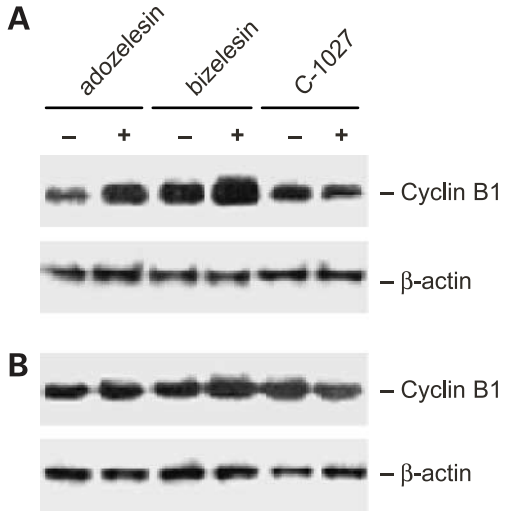


**Figure 4.** Drug-induced changes in cyclin B1 expression. **A,** wild-type HCT116 cells were treated with the indicated drug at $IC_{50}$ or vehicle control for 4 h followed by a 20 h incubation in drug-free media. Cells were extracted and equivalent mass of total cell protein was resolved by SDS-PAGE. The gels were Western blotted and blots were stained with antibodies directed against the indicated proteins. β-actin serves as a protein loading control. **B,** as in **A**, except that p21-null HCT116 cells are used.

death pathways triggered as well, the activation of different caspases was assayed in response to drug treatment. Caspase activation accompanies commitment to apoptotic cell death (26) and more than 12 different caspases exist in human cells. The pattern of caspase activation can be used to distinguish qualitatively different cell death responses (27, 28). At $IC_{50}$, total caspase activity detected in wild-type HCT116 cells 20 h postdrug treatment was substantially greater in adozelesin-treated cell extracts than in bizelesin-treated or C-1027-treated extracts (data not shown). To increase the sensitivity of subsequent caspase assays, bizelesin and C-1027 were used at $IC_{90}$ while use of adozelesin remained at $IC_{50}$. Under these conditions, bizelesin-treated cell extracts demonstrated increased caspases 2, 3/7, 5, 6, and 8–10 activity relative to vehicle-treated control. Adozelesin and C-1027 triggered the activation of a more limited subset of caspases, predominantly caspases 2, 3/7, and 9.

Because caspases can process other procaspases, the activities observed 20 h after drug treatment likely reflect initial activation of a smaller subset of caspases. An increase in caspases 2, 3/7, and 9 activity could reproducibly be detected as soon as 2 h after drug treatment. Subsequent analysis was confined to this time point to identify which of these three caspases was initially activated in response to each drug. Adozelesin at $IC_{50}$ caused the greatest relative induction of caspase activity 2 h postdrug treatment, primarily caspase-3/7 (Fig. 6). Bizelesin caused the next greatest induction of total caspase activity; significant activation of caspase-2 could be detected on drug treatment. Surprisingly, C-1027 triggered only modest increases in the activity of caspase-3/7 although C-1027 induced the greatest levels of apoptosis. This finding is consistent with the caspase-independent apoptotic cell death observed previously in MCF-7 and BEL-7402 cells on treatment with C-1027 (29).

Drug-induced caspase activation was also differentially dependent on p53 or p21 status. Consistent with the Annexin V data, adozelesin-induced or bizelesin-induced caspase activation was generally reduced in the absence of p53 while it was increased in the absence of p21 (Fig. 6). Despite significant increases in C-1027-induced apoptosis in p21-null HCT116 cells, C-1027 failed to induce significant increases in the activity of any of the caspases tested.

## Discussion

As demonstrated here and in previous studies, adozelesin, bizelesin, and C-1027 are all highly cytotoxic in HCT116 colon carcinoma cells and several other tumor cell lines. The $IC_{50}$ for adozelesin measured here using a short-term cell proliferation assay is similar to that determined previously by assaying for clonogenic survival (~0.2 nM). Hence, the cytotoxic effects of adozelesin must be rapid because short-term and long-term assays are equally sensitive in measuring them. However, the $IC_{50}$ values measured for bizelesin and C-1027 using clonogenic survival are orders of magnitude lower (picomolar) than the $IC_{50}$ values measured here (nanomolar). One possible explanation for this difference is that each assay measures a distinct subset of cytotoxic mechanisms. Support for this hypothesis is provided by the observation that low-dose bizelesin or C-1027 (picomolar) can induce a senescent phenotype (15, 30). Cellular senescence is likely to influence long-term clonogenic survival assays but not short-term cell proliferation assays. Senescence is not induced in adozelesin-treated cells (15), perhaps explaining why the

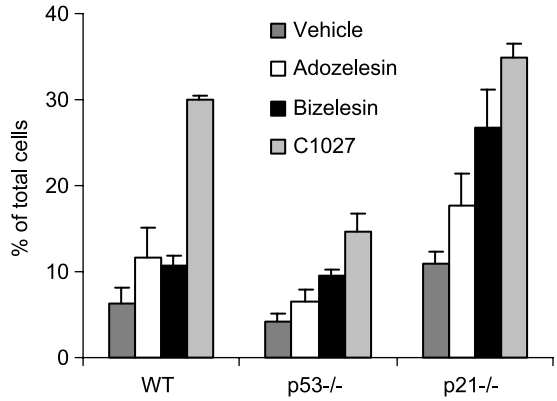


**Figure 5.** Drug-induced apoptotic cell death. Wild-type (*WT*), p53−/−, or p21−/− HCT116 cells were treated with the indicated drugs at $IC_{50}$ for 4 h followed by a 20 h incubation in drug-free media. Cells were collected and stained with Annexin V and propidium iodide. The percentage of Annexin V-positive and propidium iodide-negative cells was determined by flow cytometry. *Columns,* mean of three independent experiments; *bars,* SD.

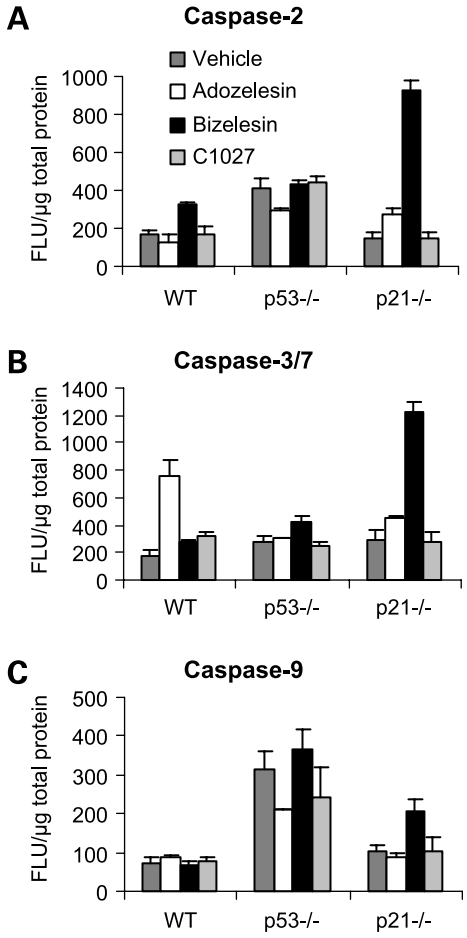


**Figure 6.** Drug-induced caspase activation. **A,** wild-type (*WT*), p53−/−, or p21−/− HCT116 cells were treated with the indicated drug at $IC_{50}$ (adozelesin), $IC_{90}$ (bizelesin and C-1027), or vehicle control for 4 h followed by a 2 h incubation in drug-free media. Cell extracts were assayed for caspase-2 activity using a fluorometric peptide substrate. The resulting fluorescence light units (*FLU*)/µg total cell protein are shown. *Columns,* mean of three independent experiments; *bars,* SD. **B,** caspase activity is measured as in **A** using a caspase-3/7 peptide substrate. **C,** caspase activity is measured as in **A** using a caspase-9 peptide substrate.

cytotoxicity of this drug is equivalent in both short-term and long-term assays. Cellular senescence, therefore, likely plays a larger role in bizelesin and C-1027 cytotoxicity than in adozelesin cytotoxicity.

While it follows that different DNA lesions produced by these drugs trigger qualitatively different cytotoxic mechanisms, it is unclear whether the different DNA lesions trigger quantitatively or qualitatively distinct cell death responses. To measure quantitative differences in the cell death response to these drugs, we have determined the relative contribution of cell cycle arrest and apoptotic cell death to short-term cytotoxicity measured at $IC_{50}$. Both adozelesin and bizelesin induce a pronounced $G_2$-M cell cycle arrest as indicated by an increase in the percentage of cells with 4N DNA content and an increase in cyclin B levels. Both of these drugs also cause a modest increase in the percentage of apoptotic cells. Hence, both cell cycle arrest and apoptotic cell death contribute to the cytotoxicity of these drugs. In contrast, C-1027 does not trigger a detectable cell cycle arrest. Rather, it induces a large increase in the percentage of apoptotic cells as measured by Annexin V staining and DNA fragmentation. C-1027 short-term cytotoxicity, therefore, is mediated primarily through apoptotic cell death. Based on these results, we conclude that the type of DNA lesion influences the relative contribution of cell cycle arrest and cell death to cytotoxicity.

In the absence of p53, both adozelesin-induced and bizelesin-induced cell death decreases while $G_2$-M checkpoint activation increases. In contrast, in the absence of p21, both adozelesin-induced and bizelesin-induced cell death increases while $G_2$-M checkpoint activation decreases. These data suggest that the relative contribution of cell cycle arrest and cell death to cytotoxicity depends not only on the DNA lesion but also on the genetic status of the cell. The net effect of these shifts in the mechanisms of cytotoxicity may be neutral with respect to the $IC_{50}$ of standard cell proliferation assays; the $IC_{50}$ of adozelesin is about the same in the presence or absence of p53, for example. Standard cytotoxicity assays, therefore, may not be good predictors of tumor cell response *in vivo*.

The apoptotic cell death response triggered by different DNA lesions may differ qualitatively and quantitatively. Comparison of the cell death pathways triggered by each drug is consistent with this hypothesis. Adozelesin and bizelesin produce similar DNA lesions (alkylation) and their cytotoxicity is mediated by both $G_2$-M cell cycle arrest and cell death. Despite these similarities, the two drugs trigger qualitatively distinct cell death pathways. Adozelesin-induced cell death is associated with greater caspase activation than bizelesin-induced cell death; a lower relative cytotoxic dose of adozelesin ($IC_{50}$) activates more total caspase activity than a higher dose ($IC_{90}$) of bizelesin. Further, bizelesin triggers detectable activation of caspase-2 whereas adozelesin does not. Although the cytotoxicity of C-1027 is primarily dependent on cell death, C-1027 does not efficiently trigger caspase activation relative to the two alkylating agents. Hence, C-1027-induced apoptosis may be relatively caspase independent.

All three of the drugs characterized in this study are highly cytotoxic. Due to the high specificity of these drugs for binding DNA, cytotoxicity is mediated almost exclusively through introduction of DNA lesions. Hence, these drugs are ideal for comparing the effects of specific DNA lesions on subsequent cytotoxic responses. While it is generally appreciated that the extent of DNA damage influences cell fate, the data presented here support the hypothesis that the nature of the DNA lesion and the genetic status of the tumor cell also has a major influence on cell fate at comparable cytotoxic dose. This is due to the qualitative and quantitative differences in the cell death and cell cycle responses triggered by distinct DNA lesions. Even closely related DNA lesions, such as those produced by adozelesin and bizelesin, can trigger qualitatively different cell death responses. Further, qualitatively distinct cell death pathways may function with different efficiencies depending on the genetic makeup of the tumor cell. As

presented here, large shifts in the mechanisms of cytotoxicity may not be detected by standard cell proliferation assays. For example, the $IC_{50}$ values of bizelesin and C-1027 are comparable, yet their mechanisms of cytotoxicity are quite different. Such differences, however, are expected to have a significant impact on clinical outcome. For example, cytotoxicity mediated by cell death is likely to have greater impact on tumor volume than cytotoxicity mediated by cell cycle arrest and/or cellular senescence. This notion emphasizes the importance of characterizing the mechanisms of drug-induced cytotoxicity and the extent of the cytotoxic response in genotoxic drug discovery efforts.

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