Cancer Chemother Pharmacol (1995) 35: 471–482 

ORIGINAL ARTICLE

Kathy S. Smith · Brian A. Folz · Earl G. Adams
Bijoy K. Bhuyan

# Synergistic and additive combinations of several antitumor drugs and other agents with the potent alkylating agent adozelesin

Received: 4 April 1994/Accepted: 22 September 1994

**Abstract** Adozelesin is a highly potent alkylating agent that undergoes binding in the minor groove of double-stranded DNA (ds-DNA) at A-T-rich sequences followed by covalent bonding with N-3 of adenine in preferred sequences. On the basis of its high-potency, broad-spectrum in vivo antitumor activity and its unique mechanism of action, adozelesin has entered clinical trial. We report herein the cytotoxicity for Chinese hamster ovary (CHO) cells of several agents, including antitumor drugs, combined with adozelesin. The additive, synergistic, or antagonistic nature of the combined drug effect was determined for most combinations using the median-effect principle. The results show that in experiments using DNA- and RNA-synthesis inhibitors, prior treatment with the DNA inhibitor aphidicolin did not affect the lethality of adozelesin. Therefore, ongoing DNA synthesis is not needed for adozelesin cytotoxicity. Combination with the RNA inhibitor cordycepin also did not affect adozelesin cytotoxicity. In experiments with alkylating agents, combinations of adozelesin with melphalan or cisplatin were usually additive or slightly synergistic. Adozelesin-tetraplatin combinations were synergistic at several different ratios of the two drugs, and depending on the schedule of exposure to drug. In experiments using methylxanthines, adozelesin combined synergistically with noncytotoxic doses of caffeine or pentoxifylline and resulted in several logs of increase in adozelesin cytotoxicity. In experiments with hypomethylating agents, adozelesin combined synergistically with 5-azacytidine (5-aza-CR) and 5-aza-2′-deoxycytidine (5-aza-2′-CdR). Combinations of adozelesin with tetraplatin or 5-aza-2′-CdR were also tested against B16 melanoma cells in vitro and were found to be additive and synergistic, respectively. The synergistic cytotoxicity to CHO cells of adozelesin combinations with tetraplatin, 5-aza-CR, or pentoxifylline was not due to increased adozelesin uptake or increased alkylation of DNA by adozelesin.

**Key words** Azodelesin combinations · Antitumor drugs · other agents

K.S. Smith · B.A. Folz · B.K. Bhuyan (✉)
Cancer and Infectious Diseases Research, The Upjohn Company, 301 Henrietta Street, Kalamazoo, MI 49001, USA

E.G. Adams
Molecular Biology Research, The Upjohn Company, Kalamazoo, MI 49001, USA

## Introduction

Combination chemotherapy remains the most effective approach for treating metastatic human malignancies. This approach is based on the assumption that each agent kills a population of cells that might be resistant to a second or third agent. The agents used in combination usually are selected on the basis of single-agent activity against a particular malignancy and the requirement that the toxicity of the agents does not overlap [12]. We report here the in vitro cytotoxicity of combinations of adozelesin, a unique base-sequence-specific alkylating agent, with several other agents.

Adozelesin (U-73975) is a synthetically derived analog of the highly potent, alkylating antitumor antibiotic CC-1065 [29]. CC-1065 undergoes nonintercalative binding in the minor groove of double-stranded DNA at A-T-rich regions followed by covalent bonding with N-3 of adenine in preferred base sequences [20, 39]. Although CC-1065 showed moderate antitumor activity in vivo, it was not evaluated clinically because it caused delayed deaths in mice at therapeutic doses [31]. Since CC-1065's receptor (the A-T-rich region in the minor groove of DNA) had been identified and the alkylating cyclopropapyrroloindole (CPI) moiety had been synthesized, it was speculated that attaching ligands of appropriate shape and length to the CPI moiety would optimize interactions with the DNA

minor groove and yield potent analogs [21, 45]. Adozelesin is one such analog that is highly cytotoxic in vitro and whose DNA alkylation properties have been described [4, 46]. It shows excellent broad-spectrum antitumor activity in preclinical in vivo models without causing delayed deaths [24]. On the basis of its superior solubility and stability in aqueous formulations, its in vivo antitumor activity, and its unique mechanism of action, adozelesin has gone to phase I clinical trial [6].

The following classes of drugs were combined with adozelesin: alkylating agents (tetraplatin, cisplatin, and melphalan), hypomethylating agents (5-azacytidine and 2′-deoxy-5-azacytidine), DNA-repair inhibitors (the methylxanthines caffeine and pentoxifylline), and DNA- and RNA- synthesis inhibitors (aphidicolin and cordycepin). The rationale for selecting these drugs for combination with adozelesin are described in Discussion. Part of this material has been presented as an abstract [38].

## Materials and methods

### Materials

Adozelesin (U-73975, mol. wt. 502.2 kDa) was synthesized at The Upjohn Company (Kalamazoo, Mich., USA) [21]. Stock solutions (1 mg/ml in dimethylacetamide) stored in the dark in a freezer were chemically stable for at least 1 year. The drug was diluted in medium immediately prior to its addition to cells. As based on cytotoxicity measurements, adozelesin was stable in medium for at least 24 h at 37°C.

Cisplatin (clinical sample containing 1 mg cisplatin, 9 mg NaCl, and 10 mg mannitol; Bristol-Myers Squibb Company, Syracuse, N.Y.) was dissolved in sterile water. DL-Tetraplatin (The Upjohn Company) was dissolved in 0.9% saline solution. 5-Azacytidine (5-aza-CR) and 2'-deoxy-5-azacytidine (5-aza-2'-CdR) as well as caffeine and pentoxifylline were dissolved in water just before use. Melphalan was dissolved in 0.01 N HCl. All drugs were further diluted in medium prior to being added to cells.

### Cell culture

The CHO/WBL (Chinese hamster ovary, clone of CHO-$K_1$) and B16 (clone of F-10) cell lines were obtained from sources described previously [4]. Culture conditions for these cell lines have been described in detail [4].

### Drug exposure and cell-survival determination

CHO cells were planted as monolayer cultures at 24–48 h before an experiment to assure exponential growth during drug exposure. Cells were exposed to drug at 37°C in their respective growth medium. For each drug concentration tested, two separate cultures were used.

After 2 h drug exposure, cell monolayers were washed with medium and harvested and cell survival was determined by a colony-forming assay as previously described [4]. The cloning efficiency of exponentially growing CHO and B16 cells ranged between 60% and 80%. The cloning efficiency of the untreated (control) cells was normalized to 100%, and that of treated cells was expressed as a percentage of control survival. The coefficient of variation (standard deviation expressed as a percentage of the mean) in determining cell survival was about 15% within each experiment. All experiments were repeated at least once.

For continuous drug exposure, cells ($2 \times 10^2$, $10^3$, or $10^4$) were plated in quadruplicate in 35-mm (6-well, Corning) dishes. Drugs were added immediately after plating of cells, which were incubated for colony formation for about 8–10 days. Cells were then stained and colonies counted [4].

### Measurement of cellular uptake of adozelesin and DNA alkylation

[$^3$H]-Adozelesin was prepared by Chemsyn Science Labs (Lenexa, Kan.). Uptake of [$^3$H]-adozelesin was measured as described previously [4] and its uptake was expressed in femtomoles of [$^3$H]-drug equivalents per $10^6$ isolated cells. To measure DNA alkylation, cellular DNA was isolated and the amount of [$^3$H]-adozelesin bound to DNA was determined [4]. DNA alkylation was expressed in [$^3$H]-drug equivalents bound per microgram of DNA.

### Macromolecule- synthesis inhibition

Incorporation of [$^3$H]-thymidine into cellular DNA or of 5-[$^3$H]-uridine into RNA was used to measure DNA or RNA synthesis as previously described [25].

### Choice of method for data analysis

A drug combination that gives an effect (cell kill) greater than that calculated for additive interaction is considered synergistic. A lower than additive response indicates antagonistic interaction. Several methods have been used to calculate the expected effect for a combination from the observed toxicity of the individual drugs if the two drugs in the combination interact in an additive manner. These methods include: (1) the fractional product [47], (2) isobologram analysis [28], and (3) median-effect analysis [7]. After considering all these methods, we decided to use median-effect analysis to interpret our data. This method has been used by many authors to analyze drug combinations [3,7,9,30,36].

In each experiment the percentage of survival was determined for each drug alone and for a series of mixtures of the two drugs combined at a constant ratio. Thus, the molar ratio of the two drugs was maintained while the absolute concentrations of the drugs changed. The survival values from the experiment were plotted as log (fa/fu) versus log (combined drug dose) = log $[(D)_1 + (D)_2]$, where *fa* is the fraction of cells killed and *fu* is the fraction of cells unaffected = 1-fa. This plot gave values for the 50% lethal dose ($LD_{50}$) for individual drugs [$(Dm)_1$ or $(Dm)_2$] and for the combination [$(Dm)_{1+2}$]. Since the ratio of the drugs in the combination is known, one can calculate the concentration of each drug present in the combination [i.e., $(D)_1$ and $(D)_2$] to yield $(Dm)_{1+2}$.

The combination index (CI) is calculated according to the following formula [9]:

$$CI = \frac{(D)_1}{(Dm)_1} + \frac{(D)_2}{(Dm)_2},$$

where $(D)_1$ and $(D)_2$ are the concentration of each drug present in the combination to give the $LD_{50}$ and $(Dm)_1$ and $(Dm)_2$ are the $LD_{50}$ concentrations of individual drug. When the drugs interact additively, CI = 1. CI < 1 indicates synergistic interaction and CI > 1 indicates antagonism [7,10]. A computer program for data analysis is available from Dr. T.C. Chou and was used in our studies.

Besides expressing the effects of the combination as CI values (Table 2), we also show the results in illustrations (Figs. 1,2,4–7) comparing the observed survival for the combination to the calculated survival expected if the drugs had interacted additively. This method of presentation shows over the total dose range the difference between the observed survival and the calculated additive survival values. To obtain the calculated additive survival values, we used the equations previously published by Chou et al. [8–10]. Those equations are reiterated herein to illustrate our exact procedure.

The median-effect equation [10] states that:

$$D = Dm[fa/1 - fa]^{1/m},$$

where $D$ is the drug dose, $Dm$ is the $LD_{50}$ dose, and $fa$ is the fractional survival. The median-effect plot linearizes the dose-survival equation shown above by plotting log $(fa/1 - fa)$ on the y-axis against log (dose) on the x-axis, in which the line has a slope of $m$ and intercepts the x-axis at log Dm. From this plot, $Dm$ can be calculated [10] as:

$$Dm = 10^{(-\text{y intercept})/m}.$$

Using the values of $Dm$ and $m$ for each individual drug, we then calculated the doses $[(Dx)_1$ and $(Dx)_2]$ needed to achieve specific survival values (e.g., $fa = 0.3$) based on the first equation. Thus, for drug 1,

$$(Dx)_1 = Dm_1[fa/1 - fa]^{1/m_1}.$$

Similarly, $(Dx)_2$ was calculated.

Next we calculated the theoretical doses of drugs 1 and 2 in the combination $[(D)_1$ and $(D)_2]$ that, if the drugs combine additively, will give the same specific survival ($fa$, 0.3). If the two drugs combine additively [7], then:

$$\frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2} = 1.$$

Since the ratio $(R)$ of the two drugs in the combination is known,

$$\frac{(D)_1}{(D)_2} = R$$

or

$$(D)_2 = \frac{(D)_1}{R}.$$

If we substitute $D_2 = \frac{D_1}{R}$ in the previous equation we get:

$$\frac{D_1}{(Dx)_1} + \frac{D_1}{R.(Dx)_2} = 1.$$

This can be solved to give:

$$(D)_1 = \frac{R \cdot (Dx)_1 \cdot (Dx)_2}{(Dx)_1 + R \cdot (Dx)_2}.$$

Similarly, $(D)_2$ can be calculated.

Thus, a theoretical additive survival curve was constructed by plotting $(D)_1$ plus $(D)_2$ (total combination dose) over a range of $fa$ values. This is the reverse of the experimental circumstance in which $fa$ is the dependent variable and $D$ is the independent variable.

## Resutls

### Lethality of individual drugs

The dose-survival relationship was determined for several drugs individually after 2 h, 24 h, or continuous exposure of CHO cells. When the dose-survival relationship follows the mass-action principle, the median-effect plot of log $(fa/fu)$ versus log dose gives a straight line with a regression coefficient $(R)$ of 0.9. The $R$ value for the dose-response curves for different drugs were consistently above 0.9 and mostly ranged between 0.95 and 1.0. Table 1 gives the $Dm$ ($LD_{50}$) values for several drugs. As expected, adozelesin was > 10,000-fold more cytotoxic than the next most cytotoxic compound, 5-aza-2′-CdR.

### Combinations with alkylating agents

Three alkylating agents (cisplatin, tetraplatin, and melphalan) were tested in two-drug combinations with adozelesin. In all cases, cell survival was determined after simultaneous exposure to the two drugs for 2 h or after continuous exposure.

**Table 1** Lethality of adozelesin and other antitumor drugs for CHO cells[a]

| Drugs | Exposure time | $LD_{50}^{b}$ $\mu M$(μg/ml) | $R$ |
|---|---|---|---|
| Adozelesin | Continuous | $4.7 \times 10^{-5}$[$(2.36 \pm 0.2) \times 10^{-5}$] | 0.98 |
| " | 2 h | $34.9 \times 10^{-5}$[$(17.5 \pm 0.8) \times 10^{-5}$] | 0.98 |
| Tetraplatin | Continuous | 4.16(1.58 ± 0.27) | 0.99 |
| " | 2 h | 27.88(10.6) | 0.93 |
| Cisplatin | Continuous | 1.67(0.5 ± 0.05) | 0.96 |
| " | 2 h | 11.64(3.49) | 0.94 |
| Melphalan | Continuous | 2.8(0.8) | 0.99 |
| 5-Aza-CR | 24 h | 14.7(3.59) | 0.99 |
| 5-Aza-2′-CR | 24 h | 1.19(0.27) | 0.96 |

[a]CHO cells were exposed to drugs for 2 h, 24 h, or continuously

[b]$LD_{50}$ is the 50% lethal dose obtained from the median-effect plot. The $R$ values (regression coefficient of the dose-response line) were also obtained from the median-effect plot. When standard deviations in $LD_{50}$ values are given, they pertain to the average from several different experiments. The $LD_{50}$ value is expressed as $\mu M$ or μg/ml (in parentheses)

**Table 2** Median-effect analysis of lethality for CHO cells of combinations of different agents with adozelesin (*Contin.* continuous exposure)

| Experiment | Adozelesin[a] (ng/ml) | 2nd Drug[a] (μg/ml) | Ratio of 2nd drug and adozelesin | Drug[b] exposure | $LD_{90}$[c] (μg/ml) | Combination index (CI)[d] at $LD_{90}$ | Conclusion |
|---|---|---|---|---|---|---|---|
| | | Tetraplatin: | | | | | |
| | 0 | 0.8–3.2 | | Contin. | 2.22 ± 0.284 | | |
| 1 | 0.01–0.025 | 0.6–1.5 | $6 \times 10^4$ | Contin. | 0.770 | 0.78 | Synergy |
| 2 | 0.015–0.03 | 0.6–1.2 | $4 \times 10^4$ | " | 0.375 | 0.89 | " |
| 3 | 0.02–0.035 | 0.57–1.0 | $2.8 \times 10^4$ | " | 0.550 | 0.82 | " |
| 4 | 0.009–0.017 | 1.2–2.27 | $13.3 \times 10^4$ | " | 1.340 | 0.85 | " |
| 5 | 0.006–0.014 | 1.2–2.8 | $20 \times 10^4$ | " | 1.275 | 0.87 | " |
| 6 | 0.008–0.02 | 1–2.5 | $1.5 \times 10^4$ | " | 1.250 | 0.9 | " |
| | 0 | 10–50 | | 2 h | 34 | | |
| 7 | 0.1–0.3 | 10–30 | $10^5$ | " | 16 | 0.82 | " |
| | | Cisplatin: | | | | | |
| | 0 | 0.3–1.3 | | Contin. | 0.89 | | |
| 8 | 0.013–0.033 | 0.26–0.66 | $2 \times 10^4$ | " | 0.44 | 1.0 | Additivity |
| 9 | 0.01–0.03 | 0.3–0.9 | $3 \times 10^4$ | " | 0.54 | 1.0 | " |
| 10 | 0.008–0.016 | 0.6–1.2 | $7.5 \times 10^4$ | " | 0.76 | 1.15 | Antagonism |
| | 0 | 1–10 | | 2 h | 8.6 | | |
| 11 | 0.14–0.3 | 2.1–4.5 | $1.5 \times 10^4$ | " | 3.3 | 0.92 | Synergy |
| | | Melphalan: | | | | | |
| | 0 | 0.25–1.25 | | Contin. | 1.6 | | |
| 12 | 0.005–0.025 | 0.25–1.25 | $5 \times 10^4$ | " | 0.86 | 0.98 | Additivity |
| 13 | 0.01–0.034 | 0.15–0.51 | $1.5 \times 10^4$ | " | 0.41 | 1.04 | " |
| | | 5-aza-CR: | | | | | |
| | 0 | 0.1–10 | | 24 h | > 10 | | |
| 14 | 0.1–0.3 | 1–3 | $10^4$ | 2–24 h | 1.02 | 0.67 | Synergy |
| 15 | 0.05–0.3 | 1.66–10 | $3.33 \times 10^4$ | " | 1.59 | 0.56 | " |
| | | 5-aza-2′-CdR: | | | | | |
| | 0 | 0.5–1.5 | | 24 h | 1.5 | | |
| 16 | 0.05–0.15 | 0.5–1.5 | $10^4$ | 2–24 h | 0.625 | 0.59 | Synergy |

[a] The range of concentrations tested for adozelesin and the second drug are shown. Each experiment in the series denotes a set of combinations in which the drugs were present in a constant ratio. For example, for experiment 1, tetraplatin:adozelesin were present at a $6 \times 10^4$: 1 ratio (see Ratio column). In experiment 1 the actual concentrations (in ng/ml) of tetraplatin + adozelesin were: 600 + 0.01, 900 + 0.015, 1200 + 0.02, 1500 + 0.025, and 1800 + 0.03. For most experiments, five such mixtures with a constant ratio were tested

[b] CHO cells were exposed continuously or for 2 h to adozelesin plus other alkylating agents (tetraplatin, cisplatin, or melphalan), with both drugs being added simultaneously. For 5-aza-CR and 5-aza-2′-CdR, cells were exposed to these drugs for 24 h prior to a 2-h exposure to adozelesin (shown as 2–24 h)

[c] $LD_{90}$ is the 90% lethal dose obtained from the median-effect plot. $LD_{90}$ for the combination = sum of concentration of each drug present individually in the combination. The *R* values (regression coefficient of the dose-response line) for this series of experiments were 0.95–1.0. The $LD_{90}$ for the second drug alone for particular exposure is given at the beginning of each set

[d] Combination index (CI) values were obtained from the median-effect analysis. These values were calculated assuming that the drugs were "mutually exclusive"

According to Chou et al. [9] when the CI = 1.0 the combination is considered additive. CI < 1 indicates synergy and CI > 1 indicates antagonism. We arbitrarily used CI = 0.95–1.05 as additive

*Tetraplatin*

The results obtained when tetraplatin and adozelesin were combined at a ratio of $6 \times 10^4$ are shown in Table 2 (experiment 1) and the actual dose-survival response is illustrated in Fig. 1. Figure 1 clearly shows the marked increase in cell kill that was observed when tetraplatin was combined with adozelesin. The CI calculated at $LD_{90}$ was < 1, indicating that the drugs interacted synergistically (Table 2). Figure 2 compares graphically the experimental survival obtained for this tetraplatin- Adozelesin combination (ratio, $6 \times 10^4$) with the calculated survival if the drugs had interacted additively. The results show that this combination was synergistic (i.e., experimental survival was less than additive survival) at drug doses that gave > 50% lethality.

Table 2 (experiments 2–6) show that the CI values were < 1.0 when tetraplatin and adozelesin were combined at several different ratios, indicating synergistic interaction. Figure 3 compares the experimental survival to the calculated additive survival for 2 h exposure to the tetraplatin-adozelesin combination and shows that the combination was synergistic. The combination had a CI value at $LD_{90}$ of 0.82 (Table 2, experiment 7).

Figure 4 shows the effect of the schedule of exposure to the drugs on the efficacy of the combination. Tetraplatin applied 4 h before adozelesin was more efficacious (CI at $LD_{90}$, 0.6) than tetraplatin added after

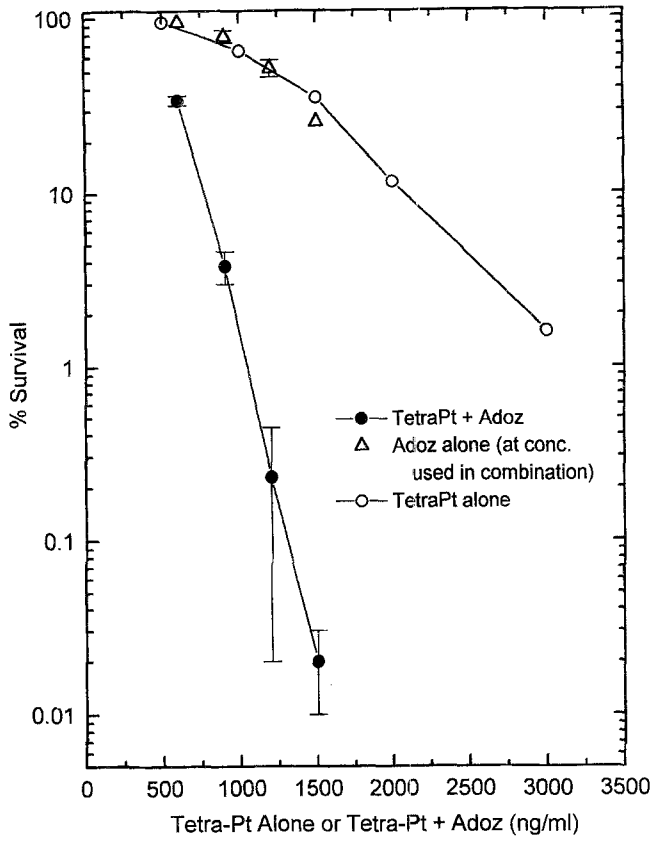



**Fig. 1** Dose-survival response of CHO cells exposed to either tetraplatin or adozelesin alone or tetraplatin + adozelesin. The x-axis denotes the concentration of either tetraplatin alone or tetraplatin + adozelesin on the same scale since the adozelesin concentration is very low ( $< 0.1$ ng/ml) as compared with the tetraplatin concentration. O——O, tetraplatin alone; ●——●, tetraplatin + adozelesin. The triangles (Δ) denote the percentage of survival for adozelesin alone at the concentration used in combination with a particular concentration of tetraplatin. For example, at a tetraplatin concentration of 1500 ng/ml we see an *open circle* (○) indicating 35.8% survival for tetraplatin alone, a *filled circle* (●) indicating 0.02% survival for tetraplatin + adozelesin, and a *triangle* (Δ) indicating 26% survival for adozelesin alone in this particular combination. Since the ratio of tetraplatin:adozelesin is $6 \times 10^4$, the adozelesin concentration is 0.025 ng/ml as used in combination with a 1500-ng/ml concentration of tetraplatin. The actual concentrations (of tetraplatin and adozelesin) for the experiment shown here are denoted in Table 2, footnote a (experiment 1)

adozelesin (CI, 0.8) or simultaneous addition of the two drugs (CI, 0.66).

*Cisplatin*

The dose-survival response observed when cells were exposed continuously to cisplatin-adozelesin (ratio, $2 \times 10^4$; experiment 8) combination is shown in Fig. 5 and is compared with the calculated survival if the drugs had interacted additively. The combination was additive except at very high lethality, where synergism was observed. Table 2 (experiments 9, 10) gives the results for combinations of cisplatin and adozelesin at various ratios. The combinations were additive to antagonistic (at $LD_{90}$, CI $\geq 1.0$).

The dose-survival response observed when cells were exposed for 2 h to the cisplatin-adozelesin combination is shown in Fig. 6 and is compared with the calculated additive survival. Figure 6 and the CI (Table 2, experiment 11) show that the combination was slightly synergistic at $LD_{90}$.

*Melphalan*

The dose-survival response of cells exposed continuously to adozelesin plus melphalan showed that the combination interacts additively (CI $\geq 1.0$; Table 2, experiments 12,13).

Combinations with the hypomethylating agents 5-aza-CR and 5-aza-2′-CdR

We essentially followed the exposure schedule of Frost et al. [18] and exposed the cells first for 24 h to 5-aza-CR or 5-aza-2′-CdR and then for 2 h to adozelesin. Cells were not washed after exposure to 5-aza-CR or 5-aza-2′-CdR and were thus exposed for the final 2 h to both adozelesin and the hypomethylating agent. The dose-survival response of cells exposed to the combinations with 5-aza-CR or 5-aza-2′-CdR are shown in Figs. 7 and 8, respectively, and are compared with the calculated additive survival. The CI values at $LD_{90}$ (Table 2, experiments 14–16) and Figs. 7 and 8 show that combinations of these agents with adozelesin were clearly synergistic.

Combinations with methylxanthines

Fingert et al. [15] showed that exposure to alkylating agents (nitrogen mustard or thio-TEPA) followed by treatment with methylxanthines (pentoxifylline or caffeine) increased the lethality of alkylating agents. In contrast to the cytotoxic concentration of other agents used in the combinations with adozelesin described above, noncytotoxic doses of methylxanthines were combined with cytotoxic concentrations of adozelesin. Since the noncytotoxic dose of methylxanthine used would not kill any cells, increased cell-kill by the combination as compared with adozelesin alone would indicate synergistic interaction.

The dose-survival response of cells exposed for 2 h to adozelesin followed by continuous exposure to caffeine or pentoxifylline is shown in Fig. 9A. The 0.5 m*M* concentration of caffeine or pentoxifylline used in the combination was noncytotoxic (data not shown). The results clearly show that combination with a noncytotoxic concentration of caffeine or pentoxifylline

Fig. 2

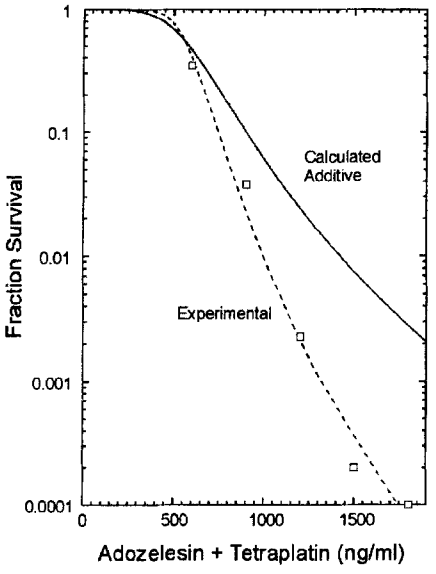



Fig. 3

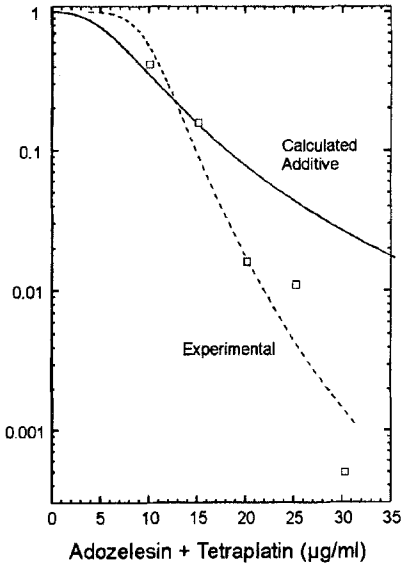



Fig. 5

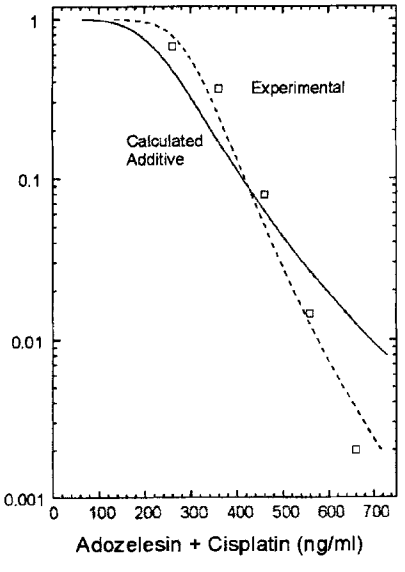



Fig. 6

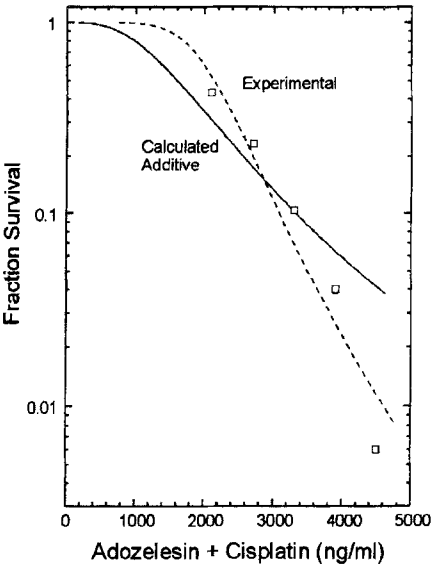



Fig. 7

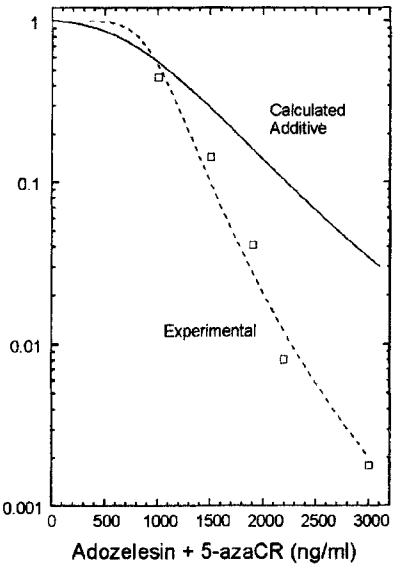
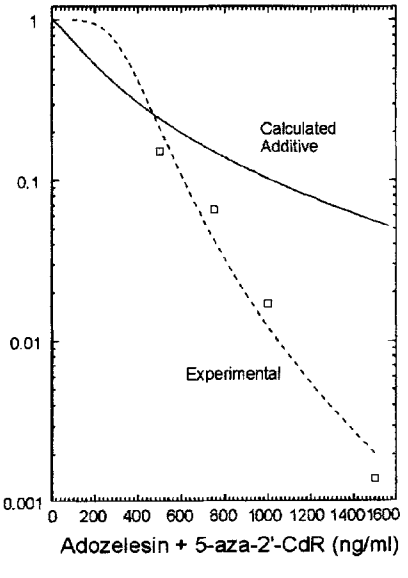



Fig. 8



increased the lethality of adozelesin by between 8- and 16-fold for the two highest adozelesin concentrations. Therefore, the combinations were strongly synergistic. Since the response to caffeine and pentoxifylline were identical and because high serum levels of pentoxifylline can be obtained in humans [1], pentoxifylline was used in further experiments.

Figure 9B shows that adozelesin's lethality increased greatly when cells were exposed continuously to adozelesin and 0.5 m*M* pentoxifylline. For example, at an adozelesin concentration of 0.035 ng/ml, 25% of the cells survived as compared with the 0.2% survival (i.e., a 2-log increase in lethality) noted for cells exposed to adozelesin plus pentoxifylline.

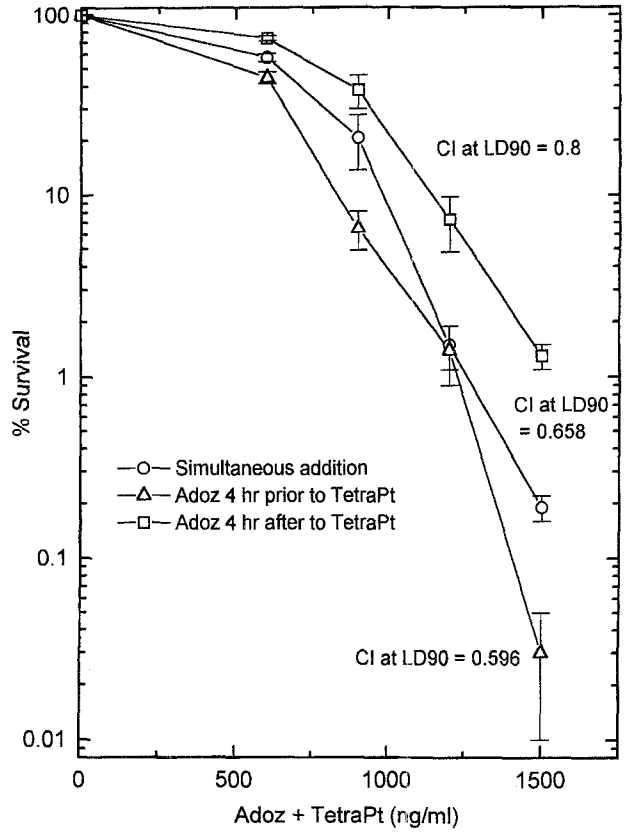



**Fig. 4** Effect of schedule of exposure to adozelesin and tetraplatin. Tetraplatin and adozelesin were either added simultaneously to CHO cells or tetraplatin was added 4 h prior to or 4 hr after adozelesin addition. The drugs were left on the cells until colony formation.

**Fig. 2,3,5–8** Comparison of experimental and calculated (for additive drug interaction) survival of cells treated with different drug combinations. The calculated additive survival values were generated using the median-effect equations of Chou et al. (7,8,10). Fractional survival is plotted against the combined dose of the two drugs. **Fig. 2** Cells continuously exposed to tetraplatin + adozelesin (experiment 1, Table 2). **Fig. 3** Cells exposed for 2 h to tetraplatin + adozelesin (experiment 7, Table 2). **Fig. 5** Cells exposed continuously to cisplatin + adozelesin (experiment 8, Table 2). **Fig. 6** Cells exposed for 2 h to cisplatin + adozelesin (experiment 11, Table 2). **Fig. 7** Cells exposed to 5-aza-CR + adozelesin (experiment 14, Table 2). **Fig. 8** Cells exposed to 5-aza-2-CdR + adozelesin (experiment 16, Table 2)

### Survival of B16 cells exposed to adozelesin plus a second agent

We next determined whether the synergistic response obtained in CHO cells would also be seen in B16 melanoma cells. Table 3 shows that the combination with tetraplatin was slightly synergistic (CI, 0.82), whereas the combination with 5-aza-2′-CdR was strongly synergistic (CI, 0.57).

Figure 10 shows the increase in cell kill observed for adozelesin plus a noncytotoxic dose of pentoxifylline as compared with adozelesin alone for two different exposure schedules (i.e., 2 h of exposure to adozelesin followed by 18 h of exposure to pentoxifylline or continuous exposure to adozelesin and pentoxifylline).

### Combination of adozelesin with inhibitors of DNA synthesis or RNA synthesis

CHO cells were initially exposed for 1 h to noncytotoxic doses of aphidicolin (10 μ*M*) or cordycepin (60 μ*M*), which caused 99% inhibition of DNA synthesis and 50% inhibition of RNA synthesis, respectively (data not shown). These cells were then exposed for 2 h to different adozelesin concentrations. We found that exposure to concentrations of aphidicolin and cordycepin that significantly inhibit DNA or RNA synthesis did not affect adozelesin's lethality (data not shown).

### Effect of tetraplatin, 5-aza-CR, or pentoxifylline on cellular uptake of adozelesin and subsequent DNA alkylation

Since combinations of adozelesin with tetraplatin, 5-aza-CR, or pentoxifylline were significantly synergistic, we determined whether these drugs would increase the cellular uptake of adozelesin and subsequent DNA alkylation. Table 4 shows that neither tetraplatin, 5-aza-CR, nor pentoxifylline significantly affected adozelesin uptake or DNA alkylation.

## Discussion

We discuss below our rationale for selecting the drugs that were combined with adozelesin and the results obtained with the combinations.

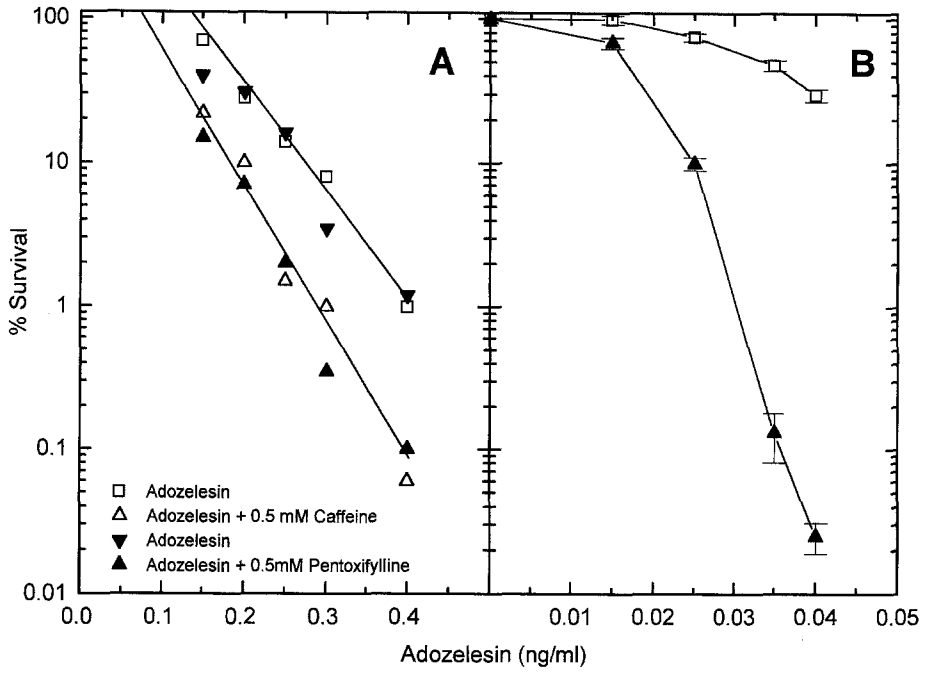



**Fig. 9A,B** Survival of CHO cells exposed to adozelesin alone or adozelesin plus caffeine or pentoxifylline. **A** Exposure for 2 h to adozelesin alone or adozelesin followed by continuous exposure to 0.5 m*M* caffeine or pentoxifylline. **B** Continuous exposure to adozelesin plus 0.5 m*M* pentoxifylline

**Table 3** Median-effect analysis of lethality for B16 cells of combinations of adozelesin with tetraplatin or 5-aza-2′CdR

| Adozelesin[a] (ng/ml) | Second Drug[a](μg/ml) | Ratio second drug to Adozelesin | Drug[b] exposure | $LD_{90}$[c] (μg/ml) | CI at[d] $LD_{90}$ | Conclusion |
|---|---|---|---|---|---|---|
| | Tetraplatin: | | | | | |
| 0 | 0.05–0.2 | | Contin. | 0.115 | | |
| 0.005–0.009 | 0.089–0.161 | $1.81 \times 10^4$ | " | 0.095 | 0.82 | Synergy |
| | 5-Aza-2′CdR | | | | | |
| 0 | 0.062–0.25 | | 24 h | 0.271 | | |
| 0.11–0.15 | 0.063–0.086 | $5.8 \times 10^2$ | 2–24 h | 0.046 | 0.57 | Synergy |

a–d see Table 2.

## Alkylating agents

Alkylating agents in combination chemotherapy provide curative treatment in several types of tumors. Our decision to combine alkylating agents with adozelesin (another alkylating agent) was based on the observation that alkylating agents are usually not cross-resistant to each other [34,40]. It had previously been assumed that alkylating agents as a class would have a common mechanism of action and, as such, would be cross-resistant to each other. However, Schabel et al. [34] showed that L1210 cells resistant to one alkylating agent showed very limited cross-resistance to other alkylating agents. Similar limited cross-resistance among alkylating agents was also reported by Frei et al. [17] and Teicher et al. [40] using human cell lines. For example, Raji cells that were 7.3-fold resistant to cisplatin were about 3-fold resistant to nitrogen mustard and carmustine (BCNU) but were not resistant to melphalan, thio-TEPA, or mitomycin C [17]. Thus, the overall impression from these studies is that significant cross-resistance among alkylating agents is the exception rather than the rule.

The relative lack of cross-resistance led to studies of combinations of alkylating agents. Schabel et al. [35] reported therapeutic synergism among alkylating agents. Synergistic cytotoxicity of different alkylating agents for epithelial ovarian cancer cells in vitro was reported by Lidor et al. [26]. In a series of studies, Teicher et al. [41] have reported on the in vitro and in vivo effects of combinations of two alkylating agents. They conducted detailed analysis of the effect of drug combinations on the cell kill both of EMT6 tumor cells in vivo and of bone-marrow granulocyte-macrophage colony-forming unit (CFU-GM) cells (as a representative normal tissue). As an example, when cyclophosphamide and melphalan were injected into EMT-6 tumor-bearing mice at 8-h intervals there was supra-additive tumor cell kill. This schedule also gave 100-fold greater tumor cell kill as compared with the toxicity to CFU-GM cells. Further impetus for combining adozelesin with alkylating agents came from the studies of Ayash et al. [2], who reported greater than additive killing of MCF-7 (human breast cancer) cells when adozelesin was combined with cisplatin, carboplatin, or melphalan. Adozelesin combinations with

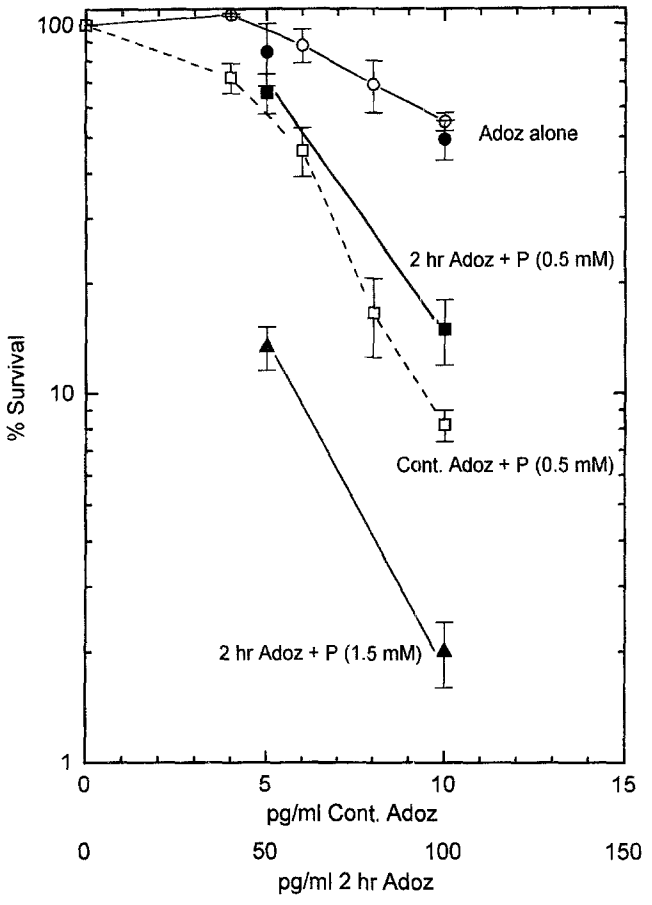



**Fig. 10** Dose-survival response of B16 cells exposed to adozelesin alone or to adozelesin + pentoxifylline. Cells were exposed for 2 h to adozelesin alone (O) or for 2 h to adozelesin and then for 18 h to 0.5 m*M* pentoxifylline (■) or 1.5 m*M* pentoxifylline (▲). Also shown are cells exposed *continuously* (*cont.*) to adozelesin alone (●) or to adozelesin + 0.5 m*M* pentoxifylline (□). Note the different scales for 2-h and continuous exposure

BCNU or thio-TEPA were additive. Finally, that autologous bone marrow transplantation can reduce alkylating-agent toxicity may make such combinations more efficacious [33].

Our results show that for combinations at different ratios of adozelesin to the second alkylating agent and under two different exposure conditions, tetraplatin was moderately synergistic at the $LD_{90}$ value, whereas cisplatin and melphalan were mostly additive. The synergism of tetraplatin-adozelesin could not be explained by greater DNA alkylation by adozelesin. However, we have not tested the obverse, i.e., whether adozelesin increased the alkylating efficacy of tetraplatin.

## Hypomethylating agents

Our choice of the hypomethylating agents 5-aza-CR and 2′-5-aza-CdR was based on the finding of Frost et al. [18] that combinations of 2′-5-aza-CdR with two alkylating agents (cisplatin and 4′-hydroperoxy-cyclophosphamide) was synergistically cytotoxic to several cell lines in vitro. Vesely [44] had also reported synergism between 2′-5-aza-CdR and cisplatin in the treatment of mouse leukemia in vivo.

It has been suggested that DNA methylation controls gene expression via conformational changes in DNA mediated by the presence or absence of specific methylcytosines. It is possible that these conformational changes in DNA could render DNA alkylation sites more accessible. This would result in synergistic toxicity of alkylating agents combined with hypomethylating agents. However, our results show that 5-aza-CR did not significantly alter DNA alkylation by adozelesin. In the studies of Frost et al. [18] the degree of DNA hypomethylation by 5-aza-CdR could not be correlated to the observed synergy. Also, 5-aza-CR plus cisplatin caused synergistic cell killing without producing demonstratable DNA hypomethylation [18]. Therefore, at present the mechanism of interaction between these alkylating agents and the hypomethylating agents leading to synergistic toxicity is undefined.

**Table 4** Effect of tetraplatin, 5-aza-CR, or pentoxifylline on uptake of adozelesin by cells and subsequent alkylation of DNA

| Expteriment | [$^3$H]-Adozelesin (ng/ml) | Tetraplatin[a] (μg/ml) | 5-Aza-CR[b] (μg/ml) | Pentoxifylline[c] (m*M*) | Cellular uptake (fmol/$10^6$cells) | DNA alkylation (fmol/μg DNA) |
|---|---|---|---|---|---|---|
| 1 | 0.05 | | | 0 | 111 ± 9 | 8 ± 2.1 |
| 1 | 0.05 | | | 1 | 96 ± 22 | 5.7 ± 1.4 |
| 2 | 0.5 | 0 | | | 686 ± 45 | 63 ± 10 |
| " | 0.5 | 50 | | | 755 ± 119 | 56 ± 10 |
| " | 0.25 | 0 | | | 370 ± 28 | 26.7 ± 0.5 |
| " | 0.25 | 25 | | | 397 ± 72 | 23.7 ± 2.9 |
| 3 | 0.2 | | 0 | | 267 ± 86 | 12 ± 3 |
| " | 0.2 | | 2 | | 308 ± 53 | 13 ± 0.5 |

[a] Cells were exposed simultaneously for 2 h to [$^3$H]-adozelesin and tetraplatin, following which the intracellular concentration of adozelesin and DNA alkylation by the drug was determined

[b] Cells were exposed to 5-aza-CR for 24 h prior to a 2-h exposure to [$^3$H]-adozelesin

[c] Cells were exposed to [$^3$H]-adozelesin and pentoxifylline for 18 h. Therefore, we used a lower concentration of adozelesin in this combination than in the other combinations

## Methylxanthines

Of all the combinations studied, those of adozelesin with caffeine or pentoxifylline seem the most interesting, partly because of the different mechanism of action of the two agents and because synergism could be shown at noncytotoxic levels of caffeine or pentoxifylline. Our decision to include methylxanthines in these studies was based on many earlier reports that pentoxifylline enhanced the cytotoxicity of alkylating agents and X-irradiation both in cell culture and in mouse tumor systems [14–16]. The cytotoxicity of alkylating agents such as thio-TEPA or nitrogen mustard for human bladder tumor cells was increased up to 10-fold when caffeine or pentoxifylline were present during the first cell cycle after exposure to the alkylating agents [16].

Teicher et al. [42] showed that the cytotoxicity-enhancing effect of pentoxifylline on MCF-7 cells in culture could depend on the alkylating agent. Pentoxifylline increased the lethality of cisplatin by 2 logs as compared with the 0.3-log increase achieved with melphalan. These investigators also observed a modest (2.9-fold) increase in the killing of tumor (FSaIIC murine fibrosarcoma) cells in vivo when pentoxifylline was combined with cisplatin, cyclophosphamide, and thio-TEPA [42].

The results we obtained using adozelesin combined with noncytotoxic doses of caffeine or pentoxifylline showed an increase of several logs in the lethality of adozelesin. We also found that the cytotoxicity-enhancing effect of caffeine and pentoxifylline was similar. However, the concentration of caffeine ( $\simeq 0.5$–$1$ m$M$) needed to enhance cytotoxicity borders on lethal serum levels in humans. Pentoxifylline, on the other hand, is used in the treatment of intermittent claudication and high serum levels are attainable [1]. Furthermore, the pentoxifylline thio-TEPA combination has completed phase I trial [13]. In the phase I trial, pentoxifylline appeared to decrease levels of cachectin (TNF) mRNA in circulating mononuclear cells in several patients, which correlated with an improved feeling of well-being in these patients. In view of these observations, we have continued further studies with the pentoxifylline-adozelesin combination, which will be the subject of a separate paper.

Caffeine and pentoxifylline have diverse biological effects due to the multiplicity of cellular targets affected. They inhibit cyclic adenosine monophosphate (AMP) phosphodiesterase [5], poly(ADP-ribose) polymerase [23], and topoisomerases I and II [37]. They can also act as anticarcinogens. For example, pentoxifylline inhibits the formation of dimethylbenzanthracene (DMBA)-induced mammary carcinomas and benign tumors [48]. However, we are interested in the ability of caffeine to potentiate the cytotoxicity of DNA-damaging agents by relaxing the $G_2$ block associated with DNA damage and, thus, not allowing enough time for repair of the damage [22]. Recent studies have elucidated the role of cdc2 kinase in promoting the progression of cells from $G_2$ to mitosis. cdc2 Kinase activity is intimately linked with changes occurring during the $G_2$ to M transition, such as chromatin condensation, nuclear envelope breakdown via laminin phosphorylation, and mitotic spindle formation. Lock [27] and Tsao et al. [43] have shown that the $G_2$ delay induced by the DNA-damaging agents etoposide and camptothecin is associated with inhibition of cdc2 kinase activity and accumulation of inactive, hyperphosphorylated cdc2. O'Connor et al. [32] also showed that cells treated with nitrogen mustard were blocked in $G_2$ due to inhibition of cdc2 kinase activity. However, the $G_2$ block was abrogated when cells were treated with nitrogen mustard plus pentoxifylline with a corresponding increase in the cdc2 kinase activity to control levels. Our studies (to be reported later) show that a similar process occurs in cells treated with adozelesin plus pentoxifylline.

## DNA- and RNA-synthesis inhibitors

Combinations with macromolecule-synthesis inhibitors were designed to determine whether such inhibition would affect the cytotoxicity of adozelesin. Several studies [11,19] have clearly shown that inhibition of DNA synthesis by aphidicolin protects cells from the cytotoxicity of topoisomerase I and II inhibitors [camptothecin and amsacrine (m-AMSA), respectively], suggesting that ongoing DNA synthesis is necessary for cell killing by these inhibitors. Inhibition of RNA synthesis by cordycepin only partially protected cells from the cytotoxicity of m-AMSA but had no effect on camptothecin's cytotoxicity. Aphidicolin and cordycepin did not affect the cytotoxicity of adozelesin.

This report clearly defines several two-drug combinations involving adozelesin that are of further interest. These include adozelesin combinations with tetraplatin, 5-aza-CR or 5-aza-2′-CdR, and pentoxifylline. We need to determine the in vivo antitumor effect of these combinations. For this purpose the studies of Teicher et al. [42] provide a good model since they have compared the survival of tumor cells with that of bone-marrow CFU-GM from the same animals. The mechanism leading to synergistic cytotoxicity for the combinations of adozelesin with tetraplatin or 5-aza-CR have not been identified. Preliminary studies suggest that the synergistic cytotoxicity of the adozelesin-pentoxifylline combination may result from the ability of pentoxifylline to abrogate the $G_2$ block induced by adozelesin.

**Acknowledgement.** We sincerely appreciate the discussions and help rendered by Dr. T-C. Chou.

## References


1. Aviado DM, Dettelbach HR (1984) Pharmacology of pentoxifylline, a hemorheologic agent used in the treatment of intermittent claudication. Angiology 35: 407
2. Ayash L, Korbut T, Herman TS, Teicher BA (1991) Combination of the minor groove binder U-73975 or the intercalator mitoxantrone with antitumor alkylating agents in MCF-7 or MCF-7/CP cells. Cancer Lett 61: 7
3. Bergerson RJ, Ingeno MJ (1987) Microbial iron chelator induced cell cycle synchronization in L1210 cells: potential in combination chemotherapy. Cancer Res 47: 6010
4. Bhuyan BK, Smith KS, Adams EG, Petzold GL, McGovren JP (1992) Lethality, DNA alkylation and cell cycle effects of adozelesin (U-73975) on rodent and human cells. Cancer Res 52: 5687
5. Bucher RW, Sutherland EW (1962) Adenosine 3',5'-phosphate in biological materials. J Biol Chem 237: 1244
6. Burris H, Earhart R, Kuhn J, Shaffer D, Smith G, Weiss G, Kasunic D, Padbury G, Campbell L, Von Hoff D (1992) A phase I trial of adozelesin, a novel DNA sequence-specific alkylating agent. Proc Am Assoc Cancer Res 33: 520
7. Chang T-T, Gulati S, Chou T-C, Colvin M, Clarkson B (1987) Comparative cytotoxicity of various drug combinations for human leukemic cells and normal hematopoietic precursors. Cancer Res 47: 119
8. Chou T-C (1991) The median effect principle and the combination index for quantitation of synergism and antagonism. In: Chou, T-C, Rideout, DC (eds) Synergism and antagonism in chemotherapy. Academic Press, New York, p 61
9. Chou T-C, Talalay P (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enz Regul 22: 27
10. Chou T-C, Talalay P (1987) Application of the median effect principle for assessment of low-dose risk of carcinogens and for the quantitation of synergism and antagonism of chemotherapeutic agents. In: Harrap, KR, Connors, TA (eds) New avenues in developmental cancer chemotherapy. Academic Press, New York, p 38
11. D'Arpa P, Beardmore C, Liu LF (1990) Involvement of nucleic acid synthesis in cell killing mechanisms of topoisomerase poisons. Cancer Res 50: 6919
12. DeVita VT (1981) The consequences of the chemotherapy of Hodgkin's disease: the 10th Annual David A. Karnofsky Lecture. Cancer 47: 1
13. Dezube BJ, Eder JP, Pardee AB (1990) Phase I trial of escalating pentoxifylline dose with constant dose of thio-TEPA. Cancer Res 50: 6806
14. Dion MD, Hussey DH, Osborne JW (1989) The effect of pentoxifylline on early and late radiation injury following fractionated irradiation in C3H mice. Int J Radiat Oncol Biol Phys 17: 101
15. Fingert HJ, Chang JD, Pardee AB (1986) Cytotoxicity, cell cycle, and chromosomal effects of methylxanthines in human tumor cells treated with alkylating agents. Cancer Res 46: 2463.
16. Fingert HJ, Pu AT, Chen Z, George PB, Alley MC, Pardee AB (1988) In vivo and in vitro enhanced antitumor effects by pentoxifylline in human cancer cells treated with thio-TEPA. Cancer Res 48: 4375
17. Frei E, Cucchi CA, Rosowsky A, Tantravahi R, Bernal S, Ervin TJ, Ruprecht RM, Haseltine WA (1985) Alkylating agent resistance: in vitro studies with human cell lines. Proc Natl Acad Sci USA 82: 2158.
18. Frost P, Abruzzese JL, Hunt BH, Lee D, Ellis M (1990) Synergistic cytotoxicity using 2'-deoxy-5-azacytidine and cisplatin or 4-hydroperoxy-cyclophosphamide with human tumor cells. Cancer Res 50: 4572
19. Holm C, Covey JM, Kerrigan D, Pommier Y (1989) Differential requirement of DNA replication for the cytotoxicity of DNA topoisomerases I and II inhibitors in Chinese hamster DC3F cells. Cancer Res 49: 6365
20. Hurley LH, Reynolds VL, Swenson DH, Petzold GL, Scahill TA (1984) Reaction of the antitumor antibiotics CC-1065 with DNA: structure of a DNA adduct with DNA sequence specificity. Science 226: 843
21. Kelly RC, Gebhard I, Wicnienski N, Aristoff PA, Johnson PD, Martin DG (1987) Coupling of cyclopropapyrroloindole (CPI) derivatives. The preparation of CC-1065, ent-CC-1065 and analogs. J Am Chem Soc 109: 6837
22. Lau CC, Pardee AB (1982) Mechanism by which caffeine potentiates lethality of nitrogen mustard. Proc Natl Acad Sci USA 79: 2942
23. Levi V, Jacobson EL, Jacobson MK (1978) Inhibition of poly(ADP-ribose)polymerase by methylated xanthines and cytokinins. FEBS Lett 88: 144
24. Li LH, Wallace TL, DeKoning T, Warpehoski M, Kelly RC, Prairie MD, Krueger WC (1987) Structure and activity relationship of several CC-1065 analogs. Invest New Drugs 5: 329
25. Li LH, Kelly RC, Warpehoski MA, McGovren JP, Gebhard I, DeKoning TF (1991) Adozelesin, a selected lead among cyclopropapyrroloindole analogs of the DNA-binding antibiotic, CC-1065. Invest New Drugs 9: 137
26. Lidor YJ, Shpall EJ, Peters WP, Bast RC (1991) Synergistic cytotoxicity of different alkylating agents for epithelial ovarian cancer. Int J Cancer 49: 704
27. Lock RB (1992) Inhibition of $p34^{cdc2}$ kinase activation, $p34^{cdc2}$ tyrosine dephosphorylation and mitotic progression of CHO cells exposed to etoposide. Cancer Res 52: 1817
28. Loewe S (1953) The problem of synergism and antagonism of combined drugs. Arzneimittelforschung 3: 285
29. Martin DG, Chidester CG, Duchamp DJ, Mizsak SA (1980) Structure of CC-1065 (NSC 298223), a new antitumor antibiotic. J Antibiot 33: 902
30. McClay EF, Albright KD, Jones JA, Eastman A, Christen RD, Howell SB (1992) Modulation of cisplatin resistance in human malignant melanoma. Cancer Res 52: 6790
31. McGovren JP, Clarke GL, Pratt EA, DeKoning TF (1984) Preliminary toxicity studies with the DNA-binding antibiotic CC-1065. J Antibiot 37: 63
32. O'Connor PM, Ferris DK, Pagano M, Draetta G, Pines J, Hunter T, Longo DL, Kohn KW (1993) $G_2$ delay induced by nitrogen mustard in human cells affects cyclin A/CDK2 and cyclin B/cdc2 kinase activities differently. J Biol Chem 268: 8298
33. Santos GW, Tutschka PJ, Brookmeyer R, Saral R, Beschorner WE, Bias WB, Braine HG, Burns WH, Elfenbein GJ, Kaizer H, Mellits D, Sensenbrenner LL, Stuart RK, Yeager AM (1983) Marrow transplantation for acute nonlymphocytic leukemia after treatment with busulfan and cyclophosphamide. N Engl J Med 309: 1347
34. Schabel FM, Trader MW, Laster WR, Wheeler GP, Witt MH (1978) Patterns of resistance and therapeutic synergism among alkylating agents. In: Schabel FM (ed) Antibiotics and chemotherapy. S. Karger, Basel, p 200
35. Schabel FM, Griswold DP, Corbett TH, Laster WR (1983) Increasing therapeutic response rates to anticancer drugs by applying basic principles of pharmacology. Pharmacol Ther 20: 282
36. Shalinsky DR, Andreef M, Howell SB (1990) Modulation of drug sensitivity by dipyridamole in multidrug resistant tumor cells in vitro. Cancer Res 50: 7537
37. Shin C, Strayer JM, Wani MA, Snakpa RM (1990) Rapid evaluation of topoisomerase inhibitors: caffeine inhibition of topoisomerases in vivo. Teratogenesis, Carcinog Mutagen 10: 41
38. Smith KS, Folz B, Adams EG, Bhuyan BK (1992) 2-Drug combinations of the potent alkylating agent adozelesin. Proc Am Assoc Cancer Res 33: 440
39. Swenson D, Li LH, Hurley LH, Rokem JS, Petzold GL, Dayton BD, Wallace TL, Lin AH, Krueger WC (1982) Mechanism of interaction of CC-1065 with DNA. Cancer Res 42: 2821

40. Teicher BA, Cuechi CA, Lee JB, Flatow JL, Rosowsky A, Frei E (1986) Alkylating agents: in vitro studies of cross-resistance patterns in human tumor cell lines. Cancer Res 46: 4379
41. Teicher BA, Holden SA, Jones SM, Eder JP, Herman TS (1989) Influence of scheduling on two-drug combinations of alkylating agents in vivo. Cancer Chemother Pharmacol 25: 161
42. Teicher BA, Holden SA, Herman T-S, Epelbaum R, Pardee AB, Dezube B (1991) Efficacy of pentoxifylline as a modulator of alkylating agent activity in vitro and in vivo. Anticancer Res 11: 1555
43. Tsao Y, D'Arpa P, Liu LF (1992) The involvement of active DNA synthesis in camptothecin-induced $G_2$ arrest: altered regulation of $p34^{cdc2}$/cyclin B. Cancer Res 52: 1823
44. Vesely J (1982) Synergistic effect of cisplatin and 5-aza-2′-deoxycytidine on mouse leukemia cells in vivo and in vitro. Int J Cancer 29: 81
45. Warpehoski MA (1986) Total synthesis of U-71184, a potent new antitumor agent modeled on CC-1065. Tet Lett 27: 4103
46. Warpehoski MA, Gebhard I, Kelly RC, Krueger WC, Li LH, McGovren JP, Prairie MD, Wicnienski N, Wierenga W (1988) Stereoelectronic factors influencing the biological activity and DNA interaction of synthetic antitumor agents modeled on CC-1065. J Med Chem 31: 590.
47. Webb JL (1963) Effect of more than one inhibitor. Enzyme and metabolic inhibitors. Academic Press, New York, p. 66, 488.
48. Wolfrom DM, Rao AR, Welsch CW. (1991) Caffeine inhibits development of benign mammary gland tumors in carcinogen-treated female Sprague-Dawley rats. Breast Cancer Res Treat 19: 269