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Sequence Selectivity of DNA Alkylation by Adozelesin and Carzelesin

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Adozelesin and carzelesin are synthetic analogues of the extremely potent antitumor antibiotic CC-1065, which alkylates N3 of adenine in a consensus sequence 5'-(A/T)(A/T)A* (A* is the site of alkylation). We have investigated the DNA sequence selectivity of adozelesin and carzelesin by thermally induced DNA strand cleavage assay using radiolabeled restriction DNA fragments. An analysis of alkylation patterns shows that the consensus sequences for carzelesin and adozelesin have been found to be 5'-(A/T)(A/T)A* and 5'-(A/T)(G/C)(A/T)A*. A new consensus sequence, 5'-(A/T)(A/T)CA*, has been observed to display an additional alkylation site for adozelesin but not for carzelesin. These results indicate that the pattern of sequence selectivity induced by carzelesin is similar but not identical to those induced by adozelesin.

Key words : Anticancer agents, DNA alkylation, Sequence selectivity, Adozelesin, Carzelesin

INTRODUCTION

Adozelesin and carzelesin are synthetic cyclopropylpyrroloindole (CPI) DNA alkylating agents derived from the natural compound CC-1065 (Li *et al.*, 1991, 1992). CC-1065 is composed of three repeating pyrroloindole subunits (A, B, and C) linked by amide bonds (Fig. 1). Subunit A contains a DNA reactive cyclopropyl group, which mediates the formation of N3 adenine covalent adducts in the minor groove of DNA (Hurley *et al.*, 1984). Subunits B and C form noncovalent interactions with DNA.

Like CC-1065, adozelesin elicits its potent cytotoxic and antitumor effects by the formation of N3 adenine covalent adducts (Lee and Gibson, 1991; Weiland and Dooley, 1991; Zsido *et al.*, 1992). Adozelesin is more potent than CC-1065 but does not produce delayed death in mice as observed with CC-1065 (McGovren *et al.*, 1984).

Carzelesin was synthesized to be an inactive prodrug, requiring chemical or enzymatic activation to the DNA reactive form (Li *et al.*, 1991). Activation of carzelesin requires two steps, i.e., (a) hydrolysis of a phenylurethane substituent to form U-76,073, followed by (b) ring closure of the chloromethyl group to form the cyclopropyl-containing DNA-reactive U-76,074 (Fig. 2). Adozelesin and carzelesin are currently being developed for clinical trials in humans by The Upjohn Company (Fleming *et al.*, 1994; Awada *et al.*, 1995).

One of the unique features of CPI agents is the ability to alkylate the adenine N3 position in a sequence-selective manner (Reynolds *et al.*, 1985; Hurley *et al.*, 1988; Hurley *et al.*, 1990; Boger *et al.*, 1994; Boger and Johnson, 1995, 1996). CPI agents form noncovalent complexes with AT-rich DNA sequences by means of hydrophobic interactions and van der Waals forces. Once bound in a noncovalent fashion, CPI agents are capable of alkylating the adenine N3 position at the 3' end of the alkylation sites. This alkylation site has been determined through thermally induced depurination and strand cleavage of labeled DNA after exposure to the agents. Initial analysis of DNA alkylation sites of CC-1065 showed that 5'-PuNTTA* and 5'-AAAAA*, where A* is the site of alkylation, are the most reactive (Reynolds *et al.*, 1985). A more detailed study has shown that a common consensus sequence 5'-(A/T)(A/T)A* is the essential sequence that conveys high reactivity (Hurley *et al.*, 1988). Adozelesin has been suggested to alkylate DNA in a manner similar to that observed for CC-1065 (Weiland and Dooley, 1991). However, recent mapping of the intracellular alkylation sites in single copy genes in human carcinoma cells by means of ligation-mediated PCR has revealed a new consensus sequence 5'-(A/T)(G/C)(A/T)A* for adozelesin (Lee *et al.*, 1994). In the case of carzelesin, the consensus sequence has not been determined yet.

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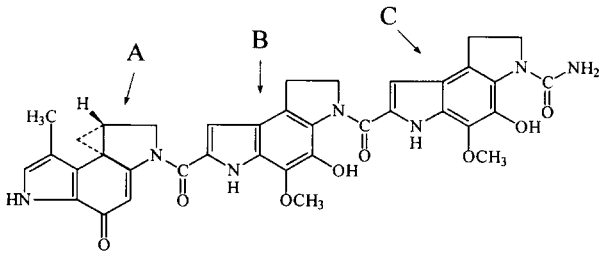
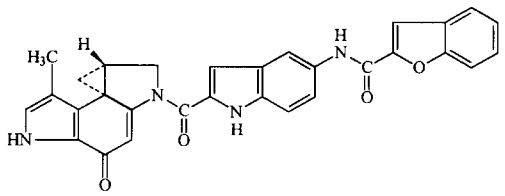




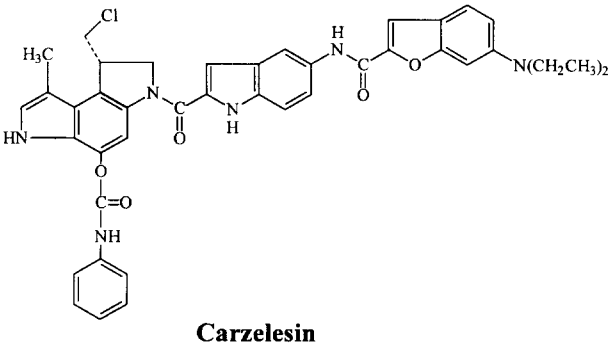
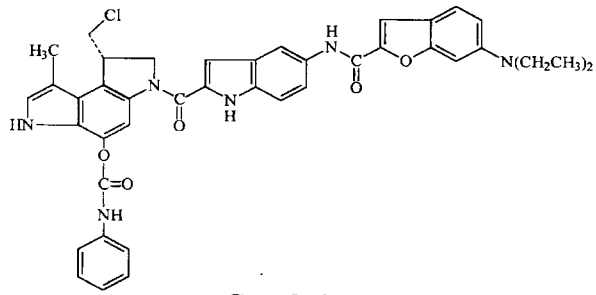
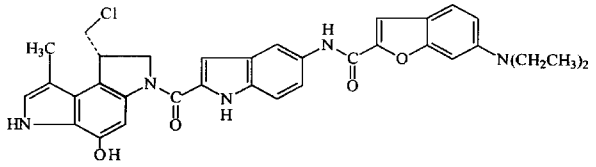


Fig. 1. Chemical structures of CC-1065, adozelesin and carzelesin.





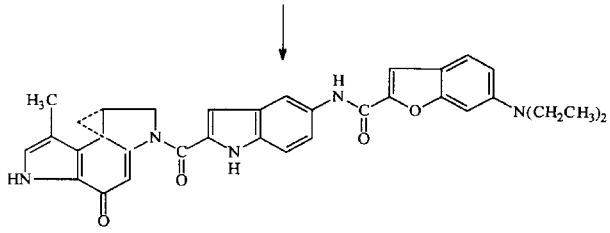


Fig. 2. Chemical conversion of carzelesin to U-76,073 and U-76,074.

In the present study, we have determined the sequence selectivity of adozelesin and carzelesin using a thermally induced DNA strand cleavage assay. An analysis of alkylation patterns shows that carzelesin and adozelesin alkylate adenine within consensus sequences 5'-(A/T)(A/T)A* and 5'-(A/T)(G/C)(A/T)A*. In addition, unique alkylation sites of adozelesin have been observed within a 5'-(A/T)(A/T)CA* sequence.

MATERIALS and METHODS

Chemicals and reagents

Adozelesin and carzelesin were generously supplied by The Upjohn Company, Kalamazoo, MI, U.S.A. Drugs were dissolved in dimethyl sulfoxide. [γ-^{32}P]ATP and [α-^{32}P]dATP were purchased from Amersham (Cleveland, OH). pBR322 DNA, ϕX174 DNA, T4 polynucleotide kinase, calf intestinal phosphate and DNA polymerase I (large fragment) were purchased from Promega (Madison, WI). Spin X centrifuge filter units were purchased from Costar (Cambridge, MA).

Preparation of end-labeled DNA

To prepare a 5' end-labeled DNA fragment of pBR 322 *Eco*RI-*Bam*HI (375 bp) or ϕX174 *Acc*I-*Dde*I (169 bp), plasmid pBR322 or ϕX174 was first linearized with the appropriate restriction enzyme, dephosphorylated with calf intestinal phosphate and 5' end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. After a second digestion with the appropriate restriction enzyme, the resulting DNA fragment was isolated by preparative electrophoresis on an 8% nondenaturing polyacrylamide gel. To prepare a 3' end-labeled DNA fragment of pBR322 *Eco*RI-*Taq*I (342 bp), plasmid pBR322 was first linearized with EcoRI and 3' end labeled with DNA polymerase I (large fragment) and [α-^{32}P]dATP. After a second digestion with TaqI, the resulting 342 bp fragment of pBR322 was isolated by preparative electrophoresis on an 8% nondenaturing polyacrylamide gel.

DNA sequencing reaction

DNA sequencing was carried out according to the

methods of Maxam and Gilbert (Maxam and Gilbert, 1980).

Reaction of drug with DNA

Each drug was mixed with radiolabeled DNA in 20 µl of 10 mM phosphate buffer, pH 7.0, at 37°C for 2 hr, and unreacted drug was removed by precipitation of DNA in ethanol, using 1 µl of tRNA (10 mg/ml) as carrier nucleic acid.

Thermally induced DNA strand cleavage assay

Aliquots of drug-induced DNA adducts were heated in 40 µl of 10 mM phosphate buffer, pH 7.0, at 92°C for 20 min. A subsequent β-elimination reaction, which hydrolyzed the phosphate backbone, was achieved by adding 4.4 µl of 10 M piperidine and then heating the mixture at 92°C for additional 20 min. The DNA was then precipitated again in ethanol, washed once with ethanol, dried under vacuum, and subjected to electrophoresis as described below.

Sequencing gel electrophoresis

Samples were resuspended in 10 µl of tracking dye containing 80% formamide, 1 mM EDTA, and xylene cyanol. After heating to 90°C for 2 min and quick cooling in an ice-water bath, equal amounts of DNA samples were loaded onto an 8% denaturing polyacrylamide gel [mono:bis (acrylamide) ratio=29:1, 8 M urea]. The sequence and alkylation sites were visualized by autoradiography. Drug alkylation sites were identified by reference to Maxam-Gilbert sequencing reactions on unmodified single-stranded DNA.

RESULTS AND DISCUSSION

Indistinguishable sequence selectivity of adozelesin and carzelesin in pBR322 *Eco*RI-*Taq*I (342 bp) DNA fragment

In order to determine the alkylation sites of adozelesin and carzelesin, thermally induced DNA strand cleavage assay was performed after drug was reacted with end-labeled restriction DNA fragments. Heat treatment of the drug-DNA adducts at neutral pH followed by hot 1 M piperidine treatment produces a second β-elimination product which displays a mobility identical to that seen with the Maxam and Gilbert sequencing reaction (Reynolds *et al.*, 1985). Bands which show high intensity upon autoradiography are considered to reflect high-affinity sites of drug alkylation.

Fig. 3 is an autoradiogram of an 8% sequencing gel showing the pattern of alkylation induced by adozelesin (Ado) and carzelesin (Car) in a 3' end-labeled pBR322 *Eco*RI-*Taq*I DNA fragment. Adozelesin and carzelesin have been found to alkylate at identical adenines with similar intensities. The highest affinity alkylation sequences observed at position numbers 1 and 2 were found to be 5'-AATAA* and 5'-AAATA*, respectively. The other alkylation sites denoted as lines coincide with the consensus sequence of 5'-(A/T)(A/T)A* for CC-1065. This result suggests that the alkylation selectivity of adozelesin and carzelesin is nearly indistinguishable.

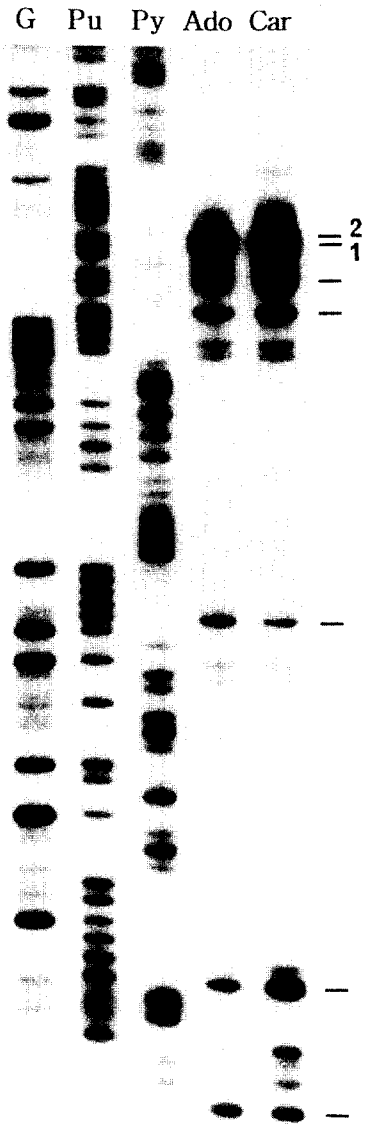


Fig. 3. Autoradiogram of an 8% sequencing gel showing the alkylation sites of adozelesin (Ado) and carzelesin (Car) in a 3' end-labeled DNA fragment of pBR322 *Eco*RI-*Taq*I (342 bp). DNA was treated with 30 nM adozelesin and 70 nM carzelesin for 2 hr followed by thermally induced DNA strand cleavage assay as described in materials and methods. Pu, purine-specific sequencing reaction; G, guanine-specific sequencing reaction; Py, pyrimidine-specific sequencing reaction.

Distinguishable sequence selectivity of adozelesin and carzelesin in pBR322 *Eco*RI-*Bam*HI and ϕX174 *Acc*I-*Dde*I DNA fragment

Adozelesin was found to alkylate human genomic DNA within 5'-(A/T)(G/C)(A/T)A* and 5'-(A/T)(A/T)A* sequences (Lee *et al.*, 1994). To find out the possibility of other alkylation sites of adozelesin and carzelesin, we have analyzed alkylation sites in additional DNA fragments of pBR322 *Eco*RI-*Bam*HI (375 bp) and ϕX174 *Acc*I-*Dde*I (169 bp).

Fig. 4 is an autoradiogram of an 8% sequencing gel showing the pattern of alkylation induced by adozelesin (Ado) and carzelesin (Car) in a 5' end-labeled pBR322 *Eco*RI-*Bam*HI DNA fragment. Although some of the sites of adenine alkylation were observed to be common for both adozelesin and carzelesin, distinguishable bands were observed at the position numbers 1, 2, 3, and 4. This strongly suggests that adenine alkylations are compound-specific. Alkylation sites of adozelesin (top) and carzelesin (bottom) showing distinguishable band intensities are represented in Fig. 5.

Carzelesin has been found to alkylate at sequences 5'-ATCTA* (position 2) and 5'-AACAA* (position 4), which can be catalogued as consensus sequence of 5'-(A/T)(G/C)(A/T)A*. This sequence is also preferred alkylation sites for adozelesin but not for CC-1065 (Lee *et al.*, 1994). In addition, the alkylation sites of carzelesin, denoted as arrows without numbers in Fig 5, coincide with the consensus sequence of 5'-(A/T)(A/T)A* for CC-1065.

Interestingly, new alkylation sites of adozelesin have been observed within 5'-TATCA* (position number 1) and 5'-TAAACA* (position number 3) sequences. Such sequences have not been reported as preferred alkylation sites for adozelesin.

In an attempt to confirm this unique sequence for adozelesin alkylation, the additional 5' end-labeled DNA fragment of ϕX174 *Acc*I-*Dde*I (169 bp) containing 5'-GAACA sequence was utilized. As shown in Fig. 6, it is evident that adozelesin and carzelesin exhibited different sequence-selective alkylation patterns. Fig. 7 represents alkylation sites of adozelesin (top) and carzelesin (bottom) in the sequence of the ϕX174 *Acc*I-*Dde*I DNA fragment. Both carzelesin and adozelesin alkylate identical adenines within 5'-GTTTA* (position number 1) and 5'-GATTA* (position number 3) sequences, which coincide with the consensus sequence of 5'-(A/T)(A/T)A* for CC-1065. Adozelesin, however, alkylates additional adenine within a 5'-GAACA* (position number 2) sequence but carzelesin does

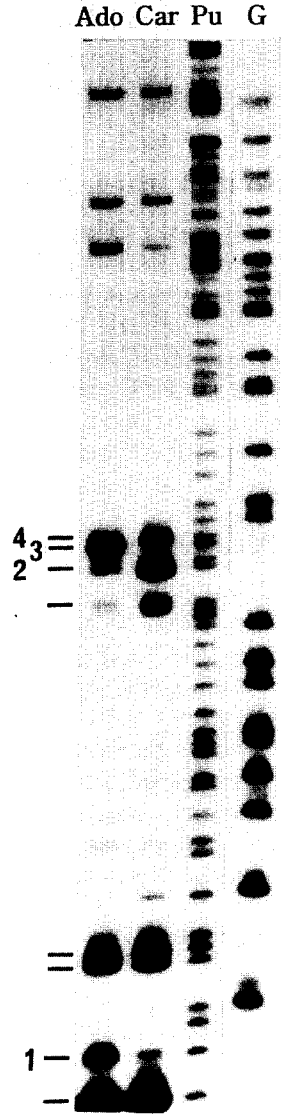


Fig. 4. Autoradiogram of an 8% sequencing gel showing the alkylation sites of adozelesin (Ado) and carzelesin (Car) in a 5' end-labeled DNA fragment of pBR322 *Eco*RI-*Bam*HI (375 bp). DNA was treated with 30 nM adozelesin and 70 nM carzelesin for 2 hr followed by thermally induced DNA strand cleavage assay as described in materials and methods. Pu, purine-specific sequencing reaction; G, guanine-specific sequencing reaction.

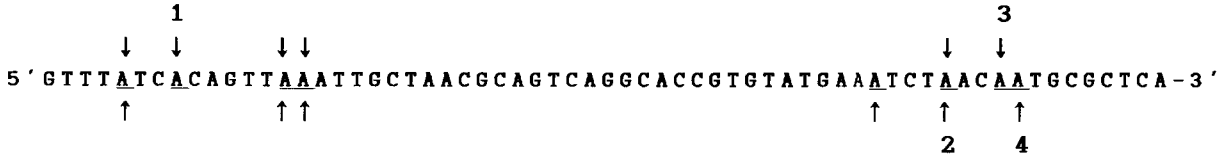


Fig. 5. Alkylation sites of adozelesin (top) and carzelesin (bottom) in the sequence of the DNA fragment of pBR322 *Eco*RI-*Bam*HI. Data were obtained from Fig. 4.

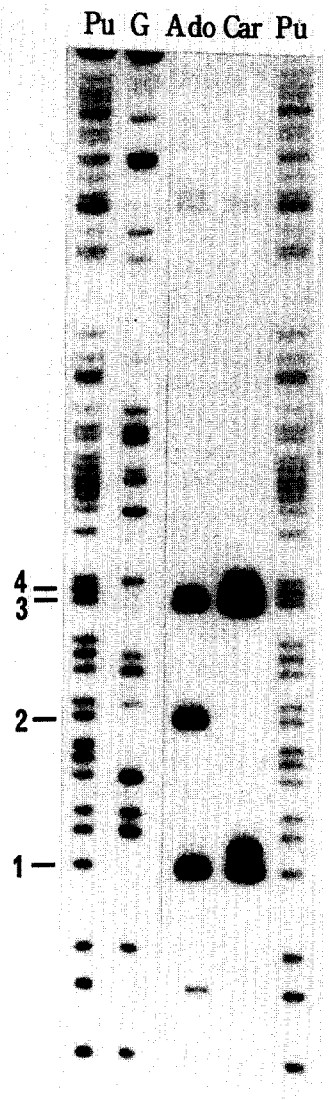


Fig. 6. Autoradiogram of an 8% sequencing gel showing the alkylation sites of adozelesin (Ado) and carzelesin (Car) in a 5' end-labeled DNA fragment of ϕX174 *Acc*I-*Dde*I (169 bp). DNA was treated with 30 nM adozelesin and 70 nM carzelesin for 2 hr followed by thermally induced DNA strand cleavage assay as described in materials and methods. Pu, purine-specific sequencing reaction; G, guanine-specific sequencing reaction.

not. In addition, the intensity of adenine alkylation observed within a 5'-GAACA* sequence is almost equivalent to those observed within 5'-GTTTA* and 5'-GATTA* sequences.

Unique sequences for adozelesin alkylation obtained from Fig. 5 and 7 are listed in Table I. The consensus sequence for adozelesin was found to be 5'-(A/T)(A/T)CA*. The adenine alkylation within 5'-(A/T)(A/T)GA* sequence has not been observed to be a preferred sequence.

In summary, the consensus sequence for adozelesin alkylation is 5'-(A/T)(A/T)A*, 5'-(A/T)(G/C)(A/T)A*, and

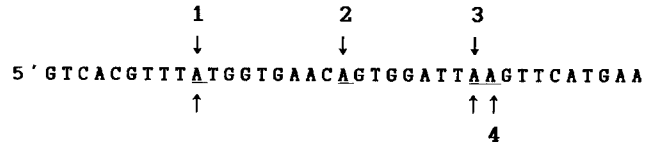


Fig. 7. Alkylation sites of adozelesin (top) and carzelesin (bottom) in the sequence of the DNA fragment of ϕX174 *Acc*I-*Dde*I. Data were obtained from Fig. 6.

Table I. Summary of unique sequences for adozelesin

Sequence	Position number
TATCA*C	1 in Fig. 4
TAACA*A	3 in Fig. 4
GAACA*G	2 in Fig. 6
(A/T)(A/T)CA*	

5'-(A/T)(A/T)CA*. The consensus sequence for carzelesin alkylation is 5'-(A/T)(A/T)A* and 5'-(A/T)(G/C)(A/T)A*. These results indicate that the consensus sequence for adozelesin alkylation is slightly different from that for carzelesin. These results are consistent with the fact that noncovalent binding interactions between the subunits B and C and DNA can be one of the important factors to mediate sequence selectivity of DNA alkylation (Reynolds *et al.*, 1985; Hurley *et al.*, 1988; Hurley *et al.*, 1990; Boger *et al.*, 1994; Boger and Johnson, 1995, 1996).

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