Depletion of Nicotinamide Adenine Dinucleotide in Normal and Xeroderma Pigmentosum Fibroblast Cells by the Antitumor Drug CC-1065†

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ABSTRACT: CC-1065 is an extremely potent antitumor antibiotic that forms a well-defined adduct with DNA in which the molecule lies within the minor groove and is covalently attached through N3 of adenine. Addition of CC-1065 to human fibroblast cells produced a prolonged depletion of the nicotinamide adenine dinucleotide (NAD) pool even at extremely low drug concentrations (0.01 μg/mL). The depletion of NAD by CC-1065 was blocked by 3-aminobenzamide, which is consistent with a NAD depletion mechanism involving poly(ADP-ribose) synthesis in response to a repair-induced DNA strand breakage event. Significantly, similar extents of NAD depletion were also evident in xeroderma pigmentosum cells of complementation groups A and D following exposure to CC-1065. Since this NAD depletion is presumably associated with repair-induced incision, the repair of CC-1065–DNA adducts can probably take place by a pathway distinct from that involved in repair of more conventional bulky DNA adducts. The prolonged depletion of NAD, even at low doses of drug, suggests that CC-1065 causes DNA damage that results in a delay or block in DNA excision repair between the excision and ligation steps.

CC-1065 is an antitumor produced by *Streptomyces zelensis* (Hanka et al., 1978). This drug is one of the most potent agents tested against L1210 cells in culture, being at least 80 and 400 times more potent on a molar basis than actinomycin D and adriamycin, respectively (Li et al., 1982). In a tumor cloning assay, CC-1065 caused a 50% decrease in the colony-forming units of tumor cells from patients with cancers of lung, breast, pancreas, ovary, etc. at a concentration of 0.1 μg/kg (Bhuyan et al., 1982). In B16 and CHO[1] cells in culture, CC-1065 is more than 100-fold more lethal than adriamycin, actinomycin D, and *cis*-diamminedichloroplatinum (Bhuyan et al., 1982). Unfortunately, CC-1065 causes lethal delayed hepatotoxicity in mice at therapeutic antineoplastic doses (McGovren et al., 1984). The chemistry, mechanism of action, and biological properties of CC-1065 have recently been reviewed (Reynolds et al., 1986).

The reaction of CC-1065 with DNA and other macromolecules has been extensively studied (Li et al., 1982; Swenson et al., 1982). CC-1065 binds covalently through N3 of adenine (Figure 1) and lies within the minor groove of DNA, overlapping with about five base pairs (Hurley et al., 1984). While binding of CC-1065 to DNA does not induce spontaneous strand breakage in DNA, upon thermal treatment of the CC-1065–DNA adduct a strand break is produced between the deoxyribose of the adenine covalent binding site and the phosphate on the 3′ side (Reynolds et al., 1985). Using thermally induced strand breakage as a means of locating CC-1065 covalent binding sites on DNA, we have discovered two distinct classes of sequences for which CC-1065 is highly specific, i.e., 5′PuNTTA and 5′AAAAA (Reynolds et al., 1985). Stereo drawings of the CC-1065–DNA adduct are predictive of the sequence specificity and are consistent with the experimentally determined nondistortive but highly stabilizing effects of drug binding on DNA (Reynolds et al., 1985; Swenson et al., 1982).

Studies of DNA alkylating agents have shown that these agents alter NAD metabolism via a mechanism, which leads to an increased rate of conversion of NAD to chromatin-associated polymers of ADP-ribose (Smulson et al., 1977; Goodwin et al., 1978; Juarez-Salinas et al., 1979; Jacobson et al., 1980), an alteration that can also lead to depletion of the cellular NAD pool. We present here a study of the effect of CC-1065 on the NAD content of human fibroblasts. Our results show that this agent is very potent in causing NAD depletion in both normal and XP fibroblasts. These data suggest that CC-1065–DNA adducts can be recognized by DNA repair enzymes other than those normally associated with bulky DNA adducts and that the CC-1065–DNA adduct causes a delay or block in DNA excision repair between the excision and ligation steps.

Materials and Methods

Materials. CC-1065 was obtained from Dr. D. Martin (Upjohn Company, Kalamazoo, MI) and was dissolved in DMF prior to use and assayed as before (Swenson et al., 1982).

Cell Culture. Normal human skin fibroblasts (CCD-45SK, ATCC CRL 1506) and xeroderma pigmentosum (XP) cell lines XP12BE (ATCC CRL 1223 complementation group A) and XP6BE (ATCC CRL 1157 complementation group D) were obtained from the American Type Culture Collection and routinely cultured as described previously (Jacobson et al., 1983). For experiments, cells were seeded in 35-mm dishes and grown with replacement of fresh medium twice per week until they reached confluence. Confluent cultures were maintained in medium with reduced serum as described pre-

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[1] Abbreviations: DMF, dimethylformamide; CHO, Chinese hamster ovary; Pu, purine; N, any nucleotide; XP, xeroderma pigmentosum; NAD, nicotinamide adenine dinucleotide; ADP, adenosine 5′-diphosphate; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine.

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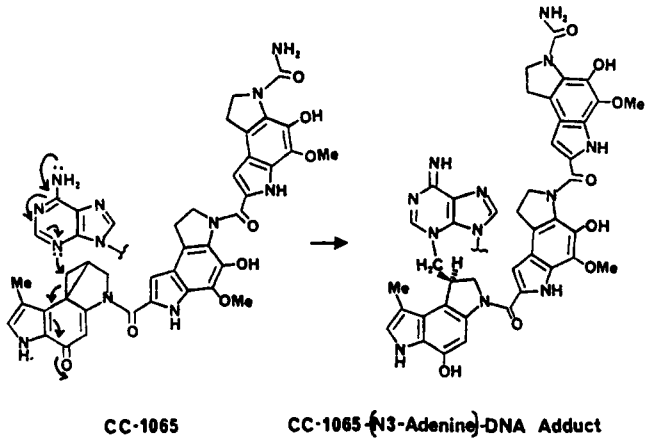

FIGURE 1: Reaction of CC-1065 with DNA to form the CC-1065-(N3-adenine)–DNA adduct.

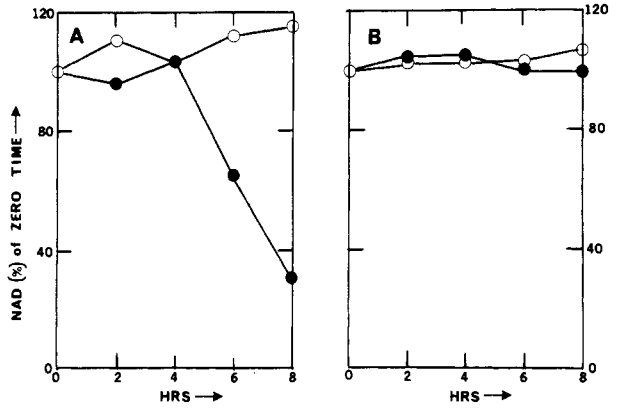

FIGURE 2: Time course of NAD content in confluent normal human fibroblasts after treatment with CC-1065 in the absence (A) or presence (B) of 10 mM 3-aminobenzamide. For dishes treated with CC-1065, a 0.5 μg/mL dose (final concentration) was added at zero time to dishes having NAD levels of 2.8×10^3 pmol/10^6 cells (A) and 3.5×10^3 pmol/10^6 cells (B). Control (○); treated with CC-1065 (●).

viously (Jacobson et al., 1983). In some experiments, cells were treated with drugs while they were still in the logarithmic phase of growth. For drug treatment, medium was replaced with fresh medium containing the indicated drugs, which had been dissolved in DMF. DMF was present at a final level of 1% in all cases and by itself had no effect on cellular NAD content.

Determination of NAD Content. The total NAD pool was extracted as described previously (Jacobson et al., 1979), and NAD content was determined by an enzymatic cycling assay described in detail elsewhere (Jacobson & Jacobson, 1976).

Results

Effect of CC-1065 on the Cellular NAD Content of Normal Human Skin Fibroblasts. CC-1065 was found to be extremely effective in causing NAD depletion in human skin fibroblasts. Figure 2 shows a time course of NAD depletion following treatment of a confluent culture of cells with 0.5 μg/mL drug. After a lag period of approximately 4 h, a progressive NAD depletion was observed. The lag was observed at doses of drug up to 40 μg/mL. This pattern of depletion was also very similar for cultures that were dividing at the time of drug treatment (data not shown).

Studies of the mechanism of NAD depletion with other DNA alkylating agents have shown that depletion is due to a rapid increase in the rate of conversion of NAD to nuclear polymers of ADP-ribose (Smulson et al., 1977; Goodwin et al., 1978; Juarez-Salinas et al., 1979; Jacobson et al., 1980). The responsible enzyme, poly(ADP-ribose) polymerase, can be inhibited in situ by benzamide and related analogues including 3-aminobenzamide (Jacobson et al., 1984). Figure 2B shows that addition of 10 mM 3-aminobenzamide to the culture medium completely prevented the NAD depletion induced by CC-1065.

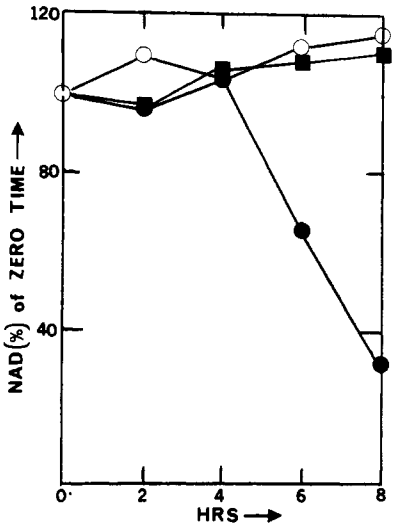

FIGURE 3: Time course of NAD content of confluent normal human fibroblasts after treatment with either CC-1065 or netropsin. CC-1065 and netropsin were added to a 0.5 μg/mL final concentration to dishes having NAD levels of 2.8×10^3 pmol/10^6 cells. Control (○); CC-1065 (●); netropsin (■).

Comparison of CC-1065 and Netropsin on NAD Content of Normal Skin Fibroblasts. Netropsin is an antiviral agent that binds noncovalently in the minor groove of DNA to AT-rich regions (Kopka et al., 1985). CC-1065 and netropsin share the same binding sites on DNA, but because of the covalent nature of the CC-1065–DNA adduct, netropsin can eventually be displaced by CC-1065 (Swenson et al., 1982). Figure 3 compares the time course of NAD content of cells following treatment with 0.5 μg/mL CC-1065 or netropsin. While NAD depletion occurred with CC-1065, netropsin had no effect on the NAD content. Thus, these data suggest that the covalent modification of DNA by CC-1065 is required to elicit NAD depletion.

Effect of CC-1065 on NAD Content in Xeroderma Pigmentosum Fibroblasts. Previous studies have shown that the occurrence of DNA strand breaks is the common factor of DNA damage that elicits poly(ADP-ribose) synthesis (Jacobson et al., 1983; Berger et al., 1980; McCurry & Jacobson, 1981). Cells homozygous for the XP genotype are grossly defective in the introduction of DNA strand breaks (and thus in the initiation of DNA excision repair) for specific types of DNA damage including pyrimidine dimers and a variety of bulky DNA adducts. XP cells are also defective in the synthesis of poly(ADP-ribose) for such lesions (Berger et al., 1980; McCurry & Jacobson, 1981). Thus, it was of interest to determine if CC-1065, which results in a bulky but nondistortive DNA adduct, would elicit NAD depletion in these cells. Parts A and B of Figure 4 compare the time course of NAD levels following CC-1065 exposure in normal and XP cells of complementation groups A (XP12BE) and D (XP6BE). The NAD content, expressed as the percentage of NAD in the cells at time zero, decreased in a similar manner in normal and XP12BE cells (Figure 4A). The absolute amount of NAD depleted from the XP12BE cells was actually twice that of normal cells since the NAD content of XP12BE was twice that of normal cells. The rate of NAD depletion of XP6BE cells

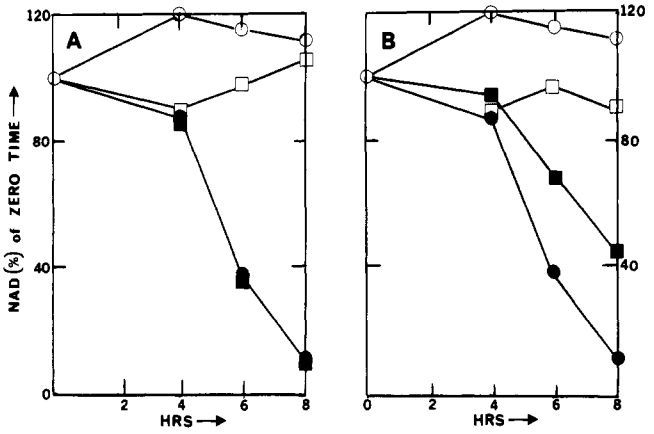

FIGURE 4: Time course of NAD content of confluent normal human fibroblasts and XP fibroblasts of complementation groups A (XP12BE) (panel A) and D (XP6BE) (panel B). CC-1065 was added to a final concentration of 0.5 μg/mL to dishes at zero time having NAD levels of 2.2×10^3 pmol/10^6 cells (normal cells), 4.9×10^3 pmol/10^6 cells (XP12BE), and 2.4×10^3 pmol/10^6 cells (XP6BE). Control and CC-1065 treated are open symbols and filled symbols, respectively; panel A, normal human fibroblasts (○ and ●) and XP12BE (□ and ■); panel B, normal human fibroblasts (○ and ●) and XP6BE (□ and ■).

Table I: Effect of CC-1065 on NAD Levels of Normal Human Fibroblasts[a]

treatment	NAD content	
	pmol/10^6 cells	%
control	864 ± 90	100
CC-1065		
0.5 μg/mL	30 ± 5.6	3.5
0.5 μg/mL, 10 mM benzamide	474 ± 85	55
0.1 μg/mL	33 ± 0.5	3.8
0.05 μg/mL	52 ± 6.8	6.0
0.01 μg/mL	362 ± 46	42
0.005 μg/mL	840 ± 14	97

[a] Confluent cultures of CCD cells were treated with the indicated doses of CC-1065, and 24 h later, NAD content was determined. Values shown are the mean values of four dishes ± standard deviations.

was somewhat less than that of normal cells but still occurred to a significant extent.

CC-1065 and Prolonged NAD Depletion. Studies with several other DNA alkylating agents have shown that NAD depletion is usually transient following DNA damage (Goodwin et al., 1978). In contrast, treatment with CC-1065 resulted in a prolonged NAD depletion even at very low doses of drug. Figure 5 shows a time course of up to 24 h, at drug doses of 0.1, 0.5, and 1.0 μg/mL. At 24 h, each of these doses resulted in an almost total depletion of NAD. Furthermore, in additional experiments, NAD depletion was detected at drug doses as low as 0.01 μg/mL after 24 h (Table I).

Discussion

The results described here show that CC-1065 is a very potent agent for effecting NAD depletion in human cells. Previous studies of the mechanism of NAD depletion using other DNA alkylating agents have provided a likely mechanism of this effect (Smulson et al., 1977; Goodwin et al., 1978; Juarez-Salinas et al., 1979; Jacobson et al., 1980, 1984; Berger et al., 1980; McCurry & Jacobson, 1981). DNA alkylation results in the appearance of DNA strand breaks either directly or indirectly due to enzymes involved in DNA excision repair. The occurrence of DNA strand breaks results in an activation of the chromatin-associated enzyme, poly(ADP-ribose) polymerase, which utilizes NAD as its substrate. Under conditions in which the cellular content of DNA strand breaks

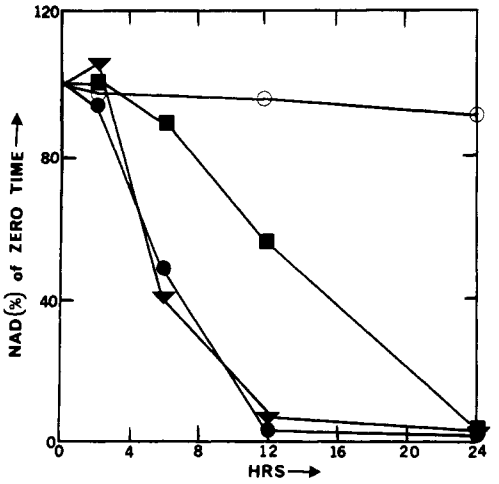

FIGURE 5: Time course of NAD content of normal human fibroblasts after treatment with various concentrations of CC-1065. Drug was added at zero time to final CC-1065 concentrations of 0 (○), 0.1 (■), 0.5 (●), and 1.0 μg/mL (▼) to flasks having NAD levels of 2.9×10^3 pmol/10^6 cells.

is sufficiently high, the rate of poly(ADP-ribose) synthesis exceeds the rate of NAD synthesis with a resulting partial or total depletion of the cellular NAD pool. Our observation that inhibitors of poly(ADP-ribose) polymerase effectively block NAD depletion induced by CC-1065 (Figure 2B) is consistent with and further supports this basic mechanism of NAD depletion by DNA alkylating agents. Further, the observation that an equivalent concentration of netropsin does not cause NAD depletion (Figure 3) suggests that it is the ability of CC-1065 to bind covalently to DNA that ultimately leads to NAD depletion.

Cells that are homozygous for the XP genotype have been shown to be grossly deficient in their ability to repair specific types of DNA lesions. These lesions include pyrimidine dimers and a variety of bulky DNA adducts. A common feature of the various complmentation groups within this genotype is the inability to cause DNA incisions prerequisite to subsequent steps of DNA excision repair. It has been previously shown that XP cells are also unable to cause NAD depletion in response to UV light (McCurry & Jacobson, 1981). Since CC-1065 causes a bulky but nondistortive DNA adduct (Reynolds et al., 1985), it was of considerable interest to determine whether XP cells could cause NAD depletion in response to this drug. Our results suggest that these cells can recognize the CC-1065–DNA adduct and can cause DNA incisions in response to this drug. Since other bulky but nondistortive DNA adducts such as that produced by the pyrrolo[1,4]benzodiazepines are not excised by XP cells (Petrusek et al., 1982), this infers that some unique feature of the CC-1065–DNA adduct must lead to recognition and subsequent incision by a repair enzyme or complex. One distinct feature of the CC-1065(N3-adenine)–DNA adduct is a positively charged purine nucleus absent in the pyrrolo-[1,4]benzodiazepine–DNA adducts which are bound through the exocyclic 2-amino group of guanine (Petrusek et al., 1981; Graves et al., 1984).

While our studies are consistent with the mechanism of NAD depletion developed from studies of other DNA alkylating agents, we have also observed unique features that may provide avenues for insight into the mechanism of the remarkable cytotoxic potency of CC-1065. First, we have observed a pronounced lag period prior to the onset of NAD depletion. This is in contrast to a very rapid onset of NAD lowering observed with other alkylating agents (Goodwin et

al., 1978; Juarez-Salinas et al., 1979; Jacobson et al., 1980). Second, the drug can cause NAD depletion at very low doses, in comparison to other alkylating agents. For example, it causes NAD depletion at doses 100–500 times less than that of the potent DNA alkylating agent MNNG (Jacobson et al., 1980). Third, the drug causes prolonged NAD depletion even at low doses, which is in marked contrast to other DNA alkylating agents where depletion is usually transient (Goodwin et al., 1978). There are two possible mechanisms that might explain the prolonged NAD depletion. First, it is possible that the drug not only causes activation of poly(ADP-ribose) synthesis but also inhibits NAD biosynthesis. This possibility has been examined for some other DNA alkylating agents but was not found to be valid (Jacobson et al., 1980). Second, it is also possible that this drug effects NAD depletion by causing a unique type of DNA damage that, while permitting recognition and DNA incisions to be made at a normal rate, does not allow DNA excision repair to be completed to the point where the incisions are resealed. Thus, poly(ADP-ribose) polymerase activation is prolonged, which in turn leads to NAD depletion.

Superficially, depletion of NAD levels caused by prolonged poly(ADP-ribose) polymerase activity due to incomplete repair of CC-1065–DNA lesions seems an attractive hypothesis to explain the high cytotoxic potency of this drug. Indeed, Berger and co-workers (Sims et al., 1983; Berger, 1985) have proposed that, at high doses of DNA damage, cytotoxicity results from NAD depletion, which in turn leads to an inability of the cell to substain normal energy metabolism and cell death. However, for CC-1065 this may not be the prime cause of cell death since in a B16 melanoma cell line a greater than 90% cell kill was found following a 2-h exposure to a 1 ng/mL concentration of CC-1065 (Bhuyan et al., 1982). Nevertheless from a mechanistic standpoint, the NAD depletion observed in our experiments can be rationalized by a block or delay in DNA excision repair between the excision and ligation steps.

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Registry No. CC-1065, 69866-21-3; NAD, 53-84-9; poly(ADP-ribose), 26656-46-2.

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