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CHARACTERIZATION OF ILLUDIN S SENSITIVITY IN DNA REPAIR-DEFICIENT CHINESE HAMSTER CELLS

UNUSUALLY HIGH SENSITIVITY OF ERCC2 AND ERCC3 DNA HELICASE-DEFICIENT MUTANTS IN COMPARISON TO OTHER CHEMOTHERAPEUTIC AGENTS

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Abstract—Illudins, novel natural products with a structure unrelated to any other known chemical, display potent *in vitro* and *in vivo* anti-cancer activity against even multi-drug resistant tumors, and are metabolically activated to an unstable intermediate that binds to DNA. The DNA damage produced by illudins, however, appears to differ from that of other known DNA damaging toxins. The sensitivity pattern of the various UV-sensitive cell lines differs from previously studied DNA cross-linking agents. Normally, the ERCC1- (excision repair cross complementing) and ERCC4-deficient cell lines are most sensitive to DNA cross-linking agents, with ERCC2-, ERCC3- and ERCC5-deficient cell lines having minimal sensitivity. With illudins the pattern is reversed, with ERCC2 and ERCC3 being the most sensitive. The sensitivity to illudins in complementation groups 1 through 3 is due to a deficiency of the ERCC1–3 gene products, as cellular drug accumulation studies revealed no differences in transport capacity or total drug accumulation. Also, a transgenic cell line in which ERCC2 activity was expressed through an expression vector regained its relative resistance to the illudins. The EM9 cell line, which displays sensitivity to monoadduct producing chemicals, was not sensitive. Thus, excision repair is involved in repair of illudin-induced damage and, unlike other anti-cancer agents, the involvement of ERCC2 and ERCC3 helicases is critical for repair to occur. The requirement for ERCC2 and ERCC3, combined with the finding that ERCC1 but not ERCC2 is upregulated in drug-resistant tumors, may explain the efficacy of illudins against drug-resistant tumors. The inhibition of DNA synthesis in cells within minutes after exposure to illudins at nanomolar concentrations may be related to the finding that the ERCC3 gene product is actually the p89 helicase component of the BTF2 (TFII) basic transcription factor and the high sensitivity of ERCC3-deficient cells to illudins.

Key words: illudin; DNA repair; UV sensitive; DNA damage; ERCC; excision repair; p89 helicase; transcription factor

The illudins are novel natural products isolated from *Omphalotus illudens* with a chemical structure unrelated to conventional chemotherapeutic agents [1]. The *in vitro* selective anti-tumor activity of these toxins was described previously [2]. With short exposure periods (<2 hr) illudins are selectively toxic towards breast, colon, lung, and ovarian adenocarcinomas as well as myeloid leukemias, whereas prolonged exposure times are required to kill other cell types [3]. This histiospecific toxicity of illudins is due to the presence of an energy-dependent system that transports the toxins into tumor cells [3]. Although native illudins are too toxic to be used *in vivo* as anti-cancer agents, our laboratory has synthesized analogs effective *in vivo* against adenocarcinomas nonresponsive to conventional agents such as cisplatin, doxorubicin HCl, mitomycin C, and taxol (paclitaxol). The analogs are also effective against (mdr)‖ tumors¶. These analogs appear to lack systemic side-effects associated with many anti-cancer agents.

Once inside tumor cells, illudins are activated metabolically to an unstable intermediate that binds to DNA. The number of illudin molecules necessary to kill a cell is several orders of magnitude less than the number of cisplatin or BCNU molecules required to kill a cell [3]. This suggests that DNA damage produced by illudins may differ from that produced by other anti-cancer agents. As presented here, UV-

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‖ Abbreviations: mdr, multiple-drug resistant; EMS, ethylmethanesulfonate; CHO, Chinese hamster ovary; ERCC, excision repair cross complementing; BCNU, *N*,*N*-bis(2-chloroethyl)-*N*-nitroso-urea or Carmustine; DMEM, Dulbecco's Modified Eagle's Medium; and TE, tris-EDTA buffer, pH 7.0.

¶ Kelner MJ, McMorris TC, Taetle R, Estes L and Hammer A, unpublished results.

sensitive cells belonging to complementation groups 2, 3 and 5 are highly sensitive to illudins. This is in contrast to cisplatin, BCNU, and mitomycin C to which only a mild sensitivity is noted in these cell lines [4].

MATERIALS AND METHODS

Cell strains, culture conditions, and cytotoxicity assays. The CHO cells were maintained in DMEM (UCSD Core Cell Culture Facility) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) at 37° in a humidified atmosphere of 95% air/5% CO_2. The parental or wild-type AA8 strain, the UV-sensitive daughter lines UV20, UV5, UV135, UV41, UV24, UV4, and the EMS-sensitive EM9 were provided by Dr. Larry Thompson (Lawrence Livermore National Laboratory, Livermore, CA). Due to the unexpected toxicity pattern obtained with illudins (described in this manuscript), the cell lines were also obtained subsequently from the ATCC (Rockville, MD), and toxicity results were confirmed. The UV61, UV40, 4PVTOR, 7PVTOR and UVS1 lines were provided by Dr. David Busch (Armed Forces Institute of Pathology, Washington, DC). Isolation, drug and UV sensitivity of these lines, as well as the UV5 derived transgenic cell line 5T4-12 with human ERCC2 expression, have been reported previously [4–19]. All cell lines were screened routinely for Mycoplasma by the UCSD Cell Culture Core Facility. The toxicity of illudin S toward the different CHO strains was determined using both a continuous 48-hr exposure with cell viability determined by trypan blue exclusion, and a 4-hr exposure followed by clonogenic assay, as described previously [2, 3].

Preparation of illudin S. O. illudens, subtype S. T. Carey 4435 (formerly *Clitocybe illudens*), was obtained from the New York Botanical Garden (New York, NY) and cultured as previously described [20]. Illudin S was isolated from the broth as previously described [1, 21]. Radiolabeled illudin S (245 mCi/mmol) was prepared by the addition to the fermentation broth of precursor [^{3}H]sodium acetate. The uptake of the radiolabeled illudin S into the different CHO strains was determined as previously described [3, 22].

DNA isolation. Genomic DNA was isolated using the G-Nome DNA Isolation Kit [BIO-101]. After exposure to tritiated illudin S, the wells were washed three times with ice-cold medium (DMEM) without fetal bovine serum, scraped, and collected into 2 mL of cell suspension solution. Then 55 μL of RNAse solution followed by 35 μL of cell lysis denaturation solution was added with mixing. After incubation at 55° for 15 min 175 μL of protease salts and 30 μL of protease mix were added. After incubation at 55° for 30 min, 1 mL of salt out mixture was added, and the solution was centrifuged for 5 min at 12,000 *g* in an Eppendorf microfuge. The supernatant was transferred to a 10-mL tube, and 50 μL of spooling salts was added, followed by mixing in 6 mL of 100% ethanol. The DNA was removed, washed twice with 70% ethanol, and resuspended in 200 μL of TE buffer. DNA content was determined by optical density at 260 nm; then 2 mL of Aquamix scintillation fluid was added (IICN Radiochemicals), and radioactivity was assayed using a Beckman LS6000 SC liquid scintillation counter, which corrects for chemical and color quenching. The radioactivity of DNA (dpm/μg) was calculated. If the three times background counts is used as the limit of detection, then 1 illudin adduct per 2 million base pairs could be detected.

To ensure that exposure to illudin S did not affect genomic DNA recovery, several different controls were performed. Cells were also exposed to non-radiolabeled illudin S, and DNA content was equivalent to cells exposed to tritiated illudin S (data not shown). Cells were also prelabeled by overnight exposure to ^{14}C-labeled thymidine, allowed to recover for 4 hr, and then exposed to either tritiated illudin S, nonradiolabeled illudin S, or no illudin S, followed by genomic DNA isolation. No difference in DNA recovery was noted as determined by recovery of carbon-14 radioactivity (data not shown). Genomic DNA was digested by *Eco*RI and electrophoresed on a 1% agarose gel. No change in distribution of DNA (by ethidium bromide fluorescence) or in radioactivity was noted (data not shown). Genomic DNA prepared by the BIO-101 method was also purified further using "elutips" (Schleicher & Schuell). No change in specific activity (radioactivity per μg of DNA) was noted (data not shown). Genomic DNA from [^{14}C]thymidine-labeled cells that were exposed to either tritiated or nontritiated illudin S (100 μg/mL, 2 hr) was also harvested with the SDS/protease-K/phenol/chloroform procedure. Again no difference in specific activity was detected (data not shown). The above controls ensure that binding of illudin to DNA did not affect recovery of genomic DNA.

Statistical analysis of data included Student's *t*-test (unpaired, two-tailed), the Tukey–Kramer Post Comparisons Test, linear regression (least squares), correlation coefficients and probability (P) values, and the analysis was performed using GraphPad Instat Software (version 2.02) (La Jolla, CA). Probability (P) values were calculated from T-values, and values of less than 0.05 were considered significant.

RESULTS

The cytotoxicity of illudin S against various DNA repair deficient complementation groups (Table 1) was first screened by using a 48-hr exposure of the different UV-sensitive CHO lines to the toxin. Based on previous alkaline elution studies, which suggested that illudins function as DNA interstrand cross-linkers*, we expected to find that the UV20 (complementation group 1)† and UV41 (complementation group 4), which are deficient in the *ERCC1* and *ERCC4* DNA-repair genes, respectively, would be the most sensitive (>10-fold) CHO sublines to illudin S and minimal sensitivity (1.0- to 2.3-fold)

* Kelner MJ, McMorris TC, Taetle R, Estes L and Hammer A, unpublished results.

† The number of complementation groups 1 and 2 was interchanged to correspond to the numbers of the complementing human gene locus names [10].

Table 1. Parental and UV-sensitive strains used in this study with their ERCC complementation group (CG), previous designation, and references

Cell line	CG	Previous designation	Reference
AA8	WT		4
UV20	1		6, 7
UV5	2		6, 7
UV24	3	56–60–21	5, 7, 14
UV41	4	361–112–10B	7, 14
UV135	5	14–47–3	13
UV61	6	6–56–37	17, 18
7PVTOR	9		15
4PVTOR	10		15
UVS1	11		23–25
EM9*	(XRCC1)		16, 26, 27
5T4-12†	(2→WT)		10,12
UV40‡		361–114–33	

* EM9 is deficient of the XRCC1 DNA repair gene and not an ERCC.

† 5T4-12 is a transgenic CHO UV5 (deficient ERCC2 homolog) cell line in which human ERCC2 is expressed through incorporation of genomic ERCC2 DNA.

‡ Unpublished strain obtained from D. Busch (AFIP). It is apparently a new mitomycin C mutant with UV, EMS, and X-ray cross-sensitivity and is not a nucleotide excision repair mutant.

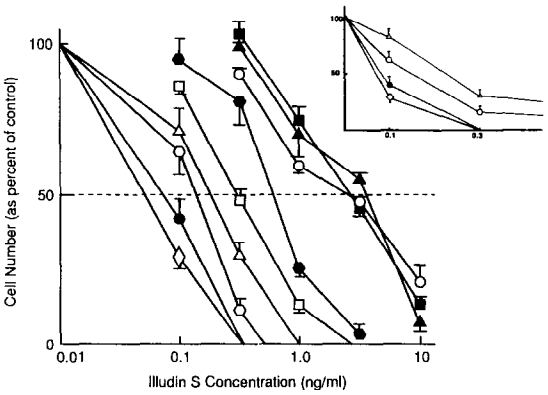

Fig. 1. Toxicity of illudin S against various CHO strains for a 48-hr continuous exposure period as determined by cell count (trypan blue exclusion, see Materials and Methods). Key: AA8 parental line (■—■), UV20 missing the ERCC1 (△—△), UV5 missing ERCC2 (●—●), UV24 missing ERCC3 (◇—◇), UV41 missing ERCC4 (○—○), UV135 missing the ERCC5 (□—□), UV61 missing ERCC6 (●—●), EM9 missing XRCC1 (▲—▲), and 5T4-12, a UV5 derived cell line with ERCC2 expressed (○—○). The other DNA-deficient repair lines had a sensitivity equivalent to the AA8 parental line, and are omitted for clarity. Values are means ± SD, N = 3–7. Inset: expanded view.

or no toxicity would be demonstrated in the UV5, UV24, and UV135 strains (ERCC2-, ERCC3-, and ERCC5-deficient strains, respectively). This would be in agreement with previous reports on toxicity of a variety of DNA alkylating and cross-linking agents to various CHO sublines [4, 8, 9, 19]. Surprisingly, this was not observed; instead, the opposite effect was seen. The UV20 and UV41 strains, as expected, were sensitive to illudins when compared with the parent AA8 strain. The sensitivity of the UV20 strain to illudin S was not as large as that previously reported with mitomycin C [7, 8, 19]. The UV24 and UV5 strains, however, were even more sensitive than UV20 and UV41 in the screening assay (Fig. 1) (UV24 vs UV20, $P < 0.01$; UV24 vs UV41, $P < 0.01$; UV5 vs UV20, $P < 0.01$; UV5 vs UV41, $P < 0.05$). Thus, the strain pattern of sensitivity was reversed. In contrast, the EM9 strain which is deficient in XRCC1, the first isolated human X-ray repair gene that is responsible for repair of single-strand breaks [9, 26, 27], was not sensitive to illudin S in agreement with previous reports on the sensitivity of various CHO strains to bifunctional alkylating agents [4, 8, 9, 19].

As cytotoxicity studies, which employ dye uptake or exclusion (such as trypan blue) to determine viability, are qualitative and not quantitative in regard to the reproductive potential of a cell, we determined the cytotoxicity of illudin S toward the different CHO strains using colony-forming assays after a 4-hr exposure to illudin S. The histiospecific toxicity of cisplatin, mitomycin C, and BCNU toward the CHO strains UV5, UV24, UV41 and UV20 was again studied as a control for DNA damaging agents. The difference between illudin S and the other three chemotherapeutic agents against complementation groups 1–4 is easily noted visually (Fig. 2). The actual IC_{50} values for illudin S against all complementation groups are shown in Table 2. The UV20 and UV41 daughter lines were sensitive (8- and 9-fold) to illudin S (Table 2). The UV5 and UV24 CHO strains were again highly sensitive (36- and 12-fold) to illudin S compared with the AA8, UV20, and UV41 lines (UV5 vs AA8 or UV20 or UV41, $P < 0.01$; UV24 vs AA8 or UV20, $P < 0.01$). The other UV-sensitive strains were less sensitive, and the EM9 strain, as expected, was not sensitive (Table 2). The UV20 and UV41 strains were highly sensitive to cisplatin, BCNU, and mitomycin C, whereas the UV5 and UV24 strains were not (Fig. 2), in agreement with the continuous exposure data and previous reports [4, 8, 9, 19].

As the illudins accumulate intracellularly by an energy-dependent transport mechanism, and the parental strain AA8 was demonstrated previously to have this transport mechanism [3], it is possible that altered sensitivity of the sublines could be due to variations in intracellular accumulation of toxin. Both the initial rate of radiolabeled toxin accumulation, and total accumulation after 4 hr, were equivalent in parental, UV5, UV20 and UV24 strains, indicating that differential uptake of toxin was not responsible for differences in cytotoxicity (Table 2). To confirm that the ERCC2 repair protein (a putative DNA helicase) was involved, the transgenic cell line 5T4-12, a UV5 derivative in which the human ERCC2 is expressed [12], was studied. There was no difference in initial illudin S uptake or in 4-hr toxin accumulation between the parent AA8, the UV5 strain, and the 5T4-12

Table 2. Illudin S toxicity, intracellular accumulation, and rate of uptake

Cell line	IC_{50}* (nM/L)	4-hr uptake† (pmol/10 million cells)	Initial rate of uptake‡ (pmol/10 million cells/hr)
AA8 (1)	28.6 ± 2.5	87 ± 9	178 ± 25
UV20 (1)	3.7 ± 0.1§	73 ± 4	192 ± 22
UV5 (2)	0.8 ± 0.1§	95 ± 2	196 ± 20
5T4-12	17.3 ± 1.6§	102 ± 3	200 ± 13
UV24 (3)	2.3 ± 0.1§	102 ± 5	178 ± 11
UV41 (4)	3.2 ± 0.1§		
UV135 (5)	3.2 ± 0.1§		
UV61 (6)	4.0 ± 0.5§		
7PVTOR (9)	>30		
4PVTOR (10)	>30		
UVS1 (11)	19 ± 1.7§		
EM9	25.9 ± 2.4		
UV40 (?)	16.7 ± 1.6§		

* Toxicity expressed as the concentration (nM/L) of illudin S that inhibits 50% of cell survival, as determined by colony-formation assays (N = 3–7; mean ± SD).

† Refers to picomoles of illudin S intracellularly accumulated in 10 million cells with a 4-hr exposure to 100 ng/mL of illudin S (N = 3–5; mean ± SD).

‡ Refers to initial rate of illudin S intracellular accumulation per 10 million cells when exposed to 100 ng/mL of illudin S for 15 min (N = 3–5; mean ± SD).

§ Statistically significant ($P < 0.01$) difference from the parental cell line AA8.

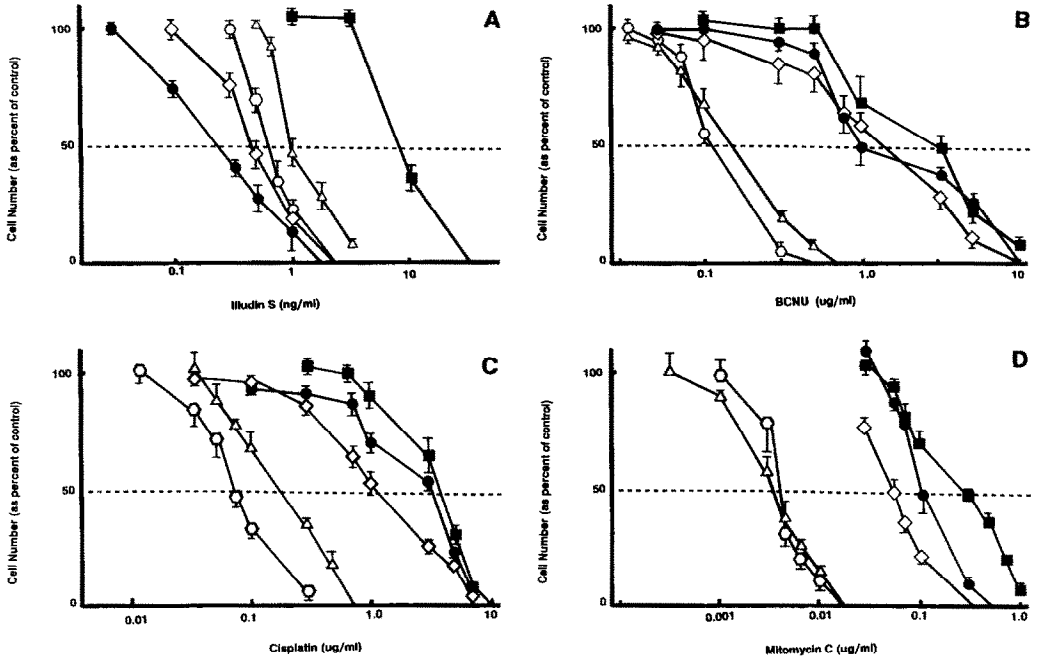

Fig. 2. Toxicity of illudin S (A), BCNU (B), cisplatin (C) and mitomycin C (D) against various CHO strains (4-hr exposure), as determined by colony-forming assays. Values are means ± SD, N = 3. Key: AA8 parental line (■—■), UV20 missing the ERCC1 (△—△), UV 5 missing ERCC2 (●—●), UV24 missing ERCC3 (◇—◇), and UV41 missing ERCC4 (○—○).

transgenic strain (Table 2). Expression of ERCC2 in the 5T4-12 strain conferred resistance to illudin S as measured by both the 48-hr (continuous exposure) screening assay and colony-forming assays (Figs. 1 and 2, and Table 2), confirming that the ERCC2 DNA repair protein was critical to the repair of illudin damage.

The covalent binding of illudin to genomic DNA was studied. The uptake and toxicity of illudins toward CHO cells and human ovarian cells are nearly identical [2, 3]. Detailed analysis of cisplatin binding to DNA in human ovarian lines has been reported previously [28]. The IC_{50} of cisplatin against the human ovarian line A2780 is 3 μM [28], similar

Table 3. Intracellular accumulation of illudin S (4-hr exposure) at the IC_{50} concentration

Cell line (CG)*	IC_{50}† (nM/L)	Molecules‡ (per cell)	Illudin adducts (per base pair)
AA8 (WT)	28.6	751,200 ± 5,800	1/210,000 ± 54,000§
UV5 (2)	1.0‖	9,600 ± 600‖	Not detectable¶
UV24 (3)	2.3‖	24,600 ± 1,200‖	Not detectable
UV20 (1)	3.7‖	57,000 ± 1,800‖	Not detectable

* Refers to complementation group (CG).

† The IC_{50} concentration for a 4-hr exposure, as determined by colony-forming assays (N = 3–7). See Table 2 for standard deviations.

‡ Average number of molecules that entered each cell during a 4-hr exposure to illudins at the 4-hr IC_{50} concentration (N = 3–5; mean ± SD).

§ Actual value based on actual experimental studies (N = 3; mean ± SD).

‖ Statistically significant ($P < 0.001$) difference from the parental cell line AA8.

¶ Limits of detection estimated to be one adduct for 2,000,000 base pairs (see Materials and Methods).

to our results (1.4 ± 0.1 μM) with CHO AA8 cells (Fig. 2). DNA binding studies revealed that one cisplatin adduct bound to every 300,000 base pairs at the IC_{50} [28].

DNA binding studies with tritiated illudin S indicated for AA8 cells that at the 4-hr exposure IC_{50} concentration, one illudin S adduct bound to every 210,000 ± 54,000 base pairs. When the UV24, UV5, and UV20 cells were exposed to tritiated illudin S at their corresponding IC_{50} values, however, no illudin S binding to DNA was detected (Table 3).

As we were unable to quantitate the amount of illudin S binding to DNA in the DNA-repair deficient cells, we determined total cellular illudin uptake at the IC_{50} value for a 4 hr exposure to illudin S as determined by colony-forming assays (Table 3). Approximately 750,000 molecules/cell of illudin S were required for 50% inhibition of the parent AA8 cells. A decrease in illudin uptake was noted in the UV5, UV20, and UV24 cells (Table 3). The UV5 cells were the most sensitive to illudin S as less than 10,000 molecules would cause a 50% inhibition in colony formation (UV5 uptake vs UV20, $P < 0.001$; UV5 vs UV24, $P < 0.001$; UV24 vs UV20, $P < 0.01$).

DISCUSSION

The DNA-damaging ability of illudins, previously suggested by indirect and direct studies [2, *], is confirmed here by the sensitivity of the UV20 and UV41 strains to the toxin. Reportedly these two CHO strains are sensitive to DNA damaging agents as no difference in sensitivity was noted between these strains and the parent AA8 line to the variety of non-DNA damaging agents, such as metabolic inhibitors, non-DNA binding carcinogens, and free radical scavengers [9].

The DNA binding studies suggest that the number of cisplatin or illudin S DNA adducts required to inhibit cloning by 50% in repair competent cells is approximately equal. The ability of illudin S to kill tumor cells at an external concentration several orders of magnitude below that of cisplatin then appears to derive from the energy-dependent transport system [3] which allows the toxins to accumulate intracellularly by several orders of magnitude (versus external concentration).* Thus, based on simple DNA binding studies, it appears that illudin S and cisplatin adducts are equivalent in regard to cell killing.

The pattern of cell killing in the DNA-repair deficient cell lines, however, suggests differences in drug action. The UV4 (UV20) and UV41 strains display about equal sensitivity to chemicals that produce large monoadducts [9]. Previous studies with a large number of DNA-cross-linking agents demonstrated that the UV4 (UV20) or UV41 strains (>8-fold), which are deficient in ERCC1 and ERCC4, respectively, show increased sensitivity to toxins, whereas there was minimal or no increased sensitivity (1.0- to 2.3-fold) for the UV5, UV24, and UV135 strains [4, 8, 9, 19]. These observations were confirmed in this study with BCNU and cisplatin. Recent reports also suggest that ERCC1 repair protein is involved in repairing cisplatin–DNA adducts [28–30]. Although the sensitivity of the UV20 (ERCC1-deficient) line to illudin S is not as great as that previously reported for mitomycin C [7, 8, 19], the pattern of sensitivity of the various CHO UV-sensitive strains to illudin S is different from that described previously for mitomycin C and other DNA damaging agents [4, 8, 9, 19]. This suggests that illudin-produced DNA damage differs from that produced by known agents.

The ERCC2 gene product, which is deficient in the UV5 strain, demonstrates marked homology to the *RAD3* yeast gene [31] and appears to function as an ATP-dependent 5′ to 3′ DNA helicase [10, 12]. Gene transfer studies indicate that ERCC2 corrects the DNA-repair defect in xeroderma pigmentosa complementation group D cells [32, 33]. The ERCC3 gene product, which is deficient in the UV24 strain [34], is also the DNA helicase that is a component of the BTF2 (TFIIH) basic transcription factor [35],

* Kelner MJ, McMorris TC, Taetle R, Estes L and Hammer A, unpublished results.

and corrects the DNA-repair defect in xeroderma pigmentosa complementation group B cells [36, 37]. The other major ERCC gene products are apparently not DNA helicases. ERCC1, based on high homology to RAD10, may function by binding to single-stranded DNA and then acting as an endonuclease [38, 39]. ERCC5, based on amino acid homology studies, apparently has no helicase motifs [40] and corrects XP-G [41, 42]. ERCC6, with high homology to the RAD16 subfamily, is believed to be either involved in dissociating protein complexes, such as RNA polymerase, from damaged DNA, or, alternatively, by scanning for blocked RNA polymerase units, guiding nuclear excision repair complexes to the site of the DNA lesion [43, 44]. Although ERCC4 has been localized to chromosome 16, the gene product and its sequence have not been identified [45, 46]. Thus, the highest sensitivity of illudins is toward cells deficient in the DNA helicases.

Why cell lines deficient in the ERCC2 and ERCC3 helicases display a higher sensitivity (compared with other DNA damaging agents) to illudins is not clear. It may be that the other enzymes involved in repairing DNA damage have difficulty recognizing or acting upon the illudin-damaged DNA without the help of the helicases. The illudin molecule may be hidden between the DNA strands (intercalating) or possibly buried in the major or minor grooves of the DNA. Unfortunately, characterization of the illudin–DNA linkage has been hindered as illudins do not spontaneously bind to DNA, even with prolonged (>24-hr) incubation. Metabolic activation of the toxin to a reactive intermediate is required [47, 48]. Thus, isolation of sufficient quantities of the illudin–DNA adduct for chemical and spectroscopy studies has not been possible.

The findings that ERCC3-deficient cells are highly sensitive to illudin S, and that ERCC3 gene product is actually the p89 helicase component of the BTF2 (TFIIH) basic transcription factor (which is required for Class II gene transcription) may explain why DNA synthesis is inhibited preferentially within minutes in cells exposed to nanomolar concentrations of illudin S. The finding that drug-resistant tumors display increased ERCC1, but not ERCC2, expression [30] may explain illudin S efficacy against these tumors. In summary, illudin-induced DNA damage appears to differ from adducts formed by conventional chemotherapeutic agents in that cell lines deficient in the putative DNA-helicases ERCC2 and ERCC3 display a higher sensitivity to this drug compared with representative mutants in other complementation groups.

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