Abstract

AS-I-145 is a novel achiral seco-amino-cyclopropylbenz[e]indolone (seco-amino-CBI) analogue of duocarmycin that has evolved from an alternative strategy of designing CC-1065/duocarmycin agents lacking the characteristic chiral center of the natural agents. The sequence specificity of this compound was assessed by a Taq polymerase stop assay, identifying the sites of covalent modification on plasmid DNA. The adenine-N3 adducts were confirmed at AT-rich sequences using a thermally induced strand cleavage assay. These studies reveal that this compound retains the inherent sequence selectivity of the related natural compounds. The AS-I-145 sensitivity of yeast mutants deficient in excision and post-replication repair (PRR) pathways was assessed. The sensitivity profile suggests that the sequence-specific adenine-N3 adducts are substrates for nucleotide excision repair (NER) but not base excision repair (BER). Single-strand ligation PCR was employed to follow the induction and repair of the lesions at nucleotide resolution in yeast cells. Sequence specificity was preserved in intact cells, and adduct elimination occurred in a transcription-coupled manner and was dependent on a functional NER pathway and Rad18. The involvement of NER as the predominant excision pathway was confirmed in mammalian DNA repair mutant cells. AS-I-145 showed good in vivo antitumor activity in the National Cancer Institute standard hollow fiber assay and was active against the human breast MDA-MD-435 xenograft when administered i.v. or p.o. Its novel structure and in vivo activity renders AS-I-145 a new paradigm in the design of novel achiral analogues of CC-1065 and the duocarmycins.

Introduction

(+)-CC-1065 and the structurally related duocarmycins, exemplified by duocarmycin SA (or (+)-DUMSA; Fig. 1), are highly potent anticancer agents, achieving cytotoxicity against mouse L1210 leukemia cells in culture in the picomolar range (1, 2). Both naturally occurring agents isolated from the fermentation broth of Streptomyces zelensis (3) and Streptomyces sp. (4), respectively, exert their biological activity through a highly sequence-selective covalent reaction of their pharmacophores with adenine-N3 in the minor groove of AT-rich sequences. The resulting drug-DNA adducts have been characterized, and the consensus sequences of interaction have been determined (5–7). CC-1065 preferentially alkylates the adenine-N3 position of the underlined residues in the 5′-PuNTTA-3′ and 5′-AAAAA-3′ sequences (Pu: purine, N; any nucleotide base) in an effectively irreversible manner (6, 8). For the duocarmycins, the high-affinity consensus sequences are 5′-(A/TAAA)-3′ and 5′-(A/TTTAPu)-3′, and these have been shown to alkylate in a reversible fashion (9). Reversible binding is implicated in “DNA walking,” until optimal sequence motifs are reached. Yatakemycin isolated from the culture of Streptomyces sp. TP-A0356 represents the newest and now most potent member of this class of compounds (10). (+)-Yatakemycin covalently reacts with the central adenine of a 5-bp AT site (e.g., 5′-AAAAA-3′), exhibiting a selectivity distinct from either (+)-CC-1065 or (+)-DUMSA (11). Like (+)-CC-1065, however, the (+)-yatakemycin–DNA alkylation is irreversible under the experimental conditions employed (11).

Despite (+)-CC-1065’s high potency and broad spectrum of antitumor activity, its further development as a clinical agent was precluded due to an irreversible, delayed hepatotoxicity in mice (12). Although (+)-DUMSA is devoid of this hepatotoxicity, it is nonetheless toxic to the bone marrow (13, 14). (+)-CC-1065 and the duocarmycins have subsequently served as leads for the design of...
novel, more efficacious agents with reduced adverse toxicity. Several of these analogues, including adozelesin (15), carzelesin (16), bizelesin (17), and KW2819 (18), have reached the clinical trial evaluation stage, highlighting the potential of such synthetic efforts. In all cases, however, myelosuppression was the most common toxicity observed (19–22).

Our laboratories have actively participated in the design and synthesis of novel synthetic, biologically active analogues of CC-1065 and the duocarmycins, including novel furano analogues of duocarmycin C1 and C2 (23). In a further attempt to develop more efficient agents and with the increasing shift in drug development from racemates to enantiomerically pure agents (24), we have more recently embarked on a program to develop achiral agents, circumventing the need for enantiomeric resolution of prepared racemic compounds. The first evidence that the chiral center present in the natural products is not absolutely required for DNA interaction, cytotoxicity, and anticancer activity came from the study of agents bearing the achiral seco-cyclopropylindolone (CI) pharmacophore. An achiral seco-hydroxy-CI-trimethoxyindole (hydroxy-CI-TMI) agent readily alkylated the adenine-N3 in the 5'-AAAAA-3' sequence, possessed an IC50 in the micromolar range against several human (K562, LS174T, PC3, and MCF-7) and murine (L1210 and P815) cancer cell lines, and showed significant activity, particularly against several solid tumor cell lines, in the National Cancer Institute (NCI) in vitro screen (25). Furthermore, this compound was relatively nontoxic to murine bone marrow cells. A seco-amino-CI-TMI analogue with comparable properties to its hydroxy counterpart was subsequently reported (26).

The natural enantiomers of cyclopropylbenz[e]indolone (CBI)-containing analogues alkylate DNA with an unaltered sequence selectivity and with enhanced cytotoxic potency over the corresponding CI counterparts due to the improved chemical stability of the CBI alkylating moiety over the authentic cyclopropylpyrroloindolone (CPI) pharmacophore (27–30). Having established that the chiral center of CC-1065 and the duocarmycins is dispensable for activity, we sought to further enhance the properties by replacing the achiral CI pharmacophore with the achiral CBI alkylationsubunit. The synthesis and initial evaluation of AS-I-145 (Fig. 1), an achiral seco-amino-CBI-TMI agent, has been reported (31), confirming the expected enhanced potency and establishing that the achiral CBI alkylating moiety constitutes a feasible substitute to the established chiral pharmacophore, offering a template for alternative drug design of novel active analogues.

Both budding yeast and mammalian cells with defined defects in specific DNA repair pathways have served as model systems for the elucidation of the cellular responses to minor groove-targeted adducts (32, 33). The ability of the cells to recognize and repair such highly sequence-specific adducts is a critical determinant of cytotoxicity, with minor groove lesions reported to be more cytotoxic than those induced by more conventional major groove agents (33, 34). In this paper, the sequence-selective DNA adducts induced by AS-I-145 are characterized, the mechanisms involved in the repair of the relevant minor groove adducts are investigated in yeast and mammalian cells, and data confirming in vivo antitumor activity are presented.

Materials and Methods

Chemicals

The synthesis of AS-I-145 has been described previously (31). The compound was dissolved in dimethylacetamide (DMA; Sigma) at a 10 mmol/L stock concentration and stored at −20°C until immediately before use. Dilutions were freshly prepared for each experiment in 1× 25 mmol/L triethanolamine, 1 mmol/L EDTA (TEOA) buffer. Adozelesin was a kind gift from Dr. Robert Kelly (Pharmacia & Upjohn, Kalamazoo, MI).

Measurement of Sequence Selectivity of Interaction to Naked DNA

Taq Polymerase Stop (Termination) Assay. The sites of covalent modifications induced by AS-I-145 on the bottom strand of the pUC18 plasmid DNA (Sigma) were identified using the Taq polymerase stop assay developed by Hartley et al. (35). The plasmid was linearized with the restriction enzyme HindIII (Promega) to provide a downstream block for the polymerase and was linearly amplified with the singly radiolabeled primer 5'-CTCACTCAAAGGCCGTTAATAC-3', which binds to the sequence region 749 to 769 of the bottom strand of pUC18. The experimental conditions employed and procedures followed were as described in ref. 35. Briefly, 0.5 µg of plasmid DNA per experimental point was reacted with drug in aqueous TEOA buffer [25 mmol/L triethanolamine, 1 mmol/L EDTA (pH, 7.2)] for 5 h at 37°C, in a total volume of 50 µL.
50 µL. The primer was 5-end labeled with [γ-32P] ATP (5000 Ci/mmol, Amersham) using T4 polynucleotide kinase (Invitrogen). The PCR linear amplification was carried out in a total volume of 100 µL per sample containing 0.5 µg DNA (50 µL), 5 pmol of the radiolabeled primer, 250 µmol/L of each deoxynucleotide triphosphate (dNTP, Amersharm Pharmacia), 1 unit of Taq polymerase (Promega), the supplied 10× reaction buffer [when diluted 1:10, it has a composition of 10 mmol/L Tris-HCl (pH, 9.0 at 25°C), 50 mmol/L KCl, and 0.1% Triton X-100], 2.5 mmol/L MgCl2, and 0.01% gelatin. PCR was carried out using cycling conditions consisting of an initial 4-min denaturation step at 94°C, 1 min at 60°C and 1 min at 72°C, for a total of 30 cycles. After primer extension, the samples were ethanol and sodium acetate precipitated, washed with 70% ethanol, and dried by lyophilization. Samples were finally resuspended in 5 µL formamide loading buffer and subjected to electrophoresis.

**Thermal Cleavage Assay.** The thermally induced cleavage assay, first described by Reynolds et al. (6), was used to probe purine-N3 alkylations on the same region of the pUC18 as the one examined with the Taq polymerase assay. The 208-bp probe subjected to drug modification was PCR amplified and defined by the primer mentioned above and a second primer, 5'-TGGTATCTTTATAGTC-CGTGTC-3', which binds to the top strand of the plasmid at positions 956–935. In this case, the second primer extending the bottom strand was the radiolabeled one. Both oligonucleotide primers were synthesized by MWG (Ebersberg, Germany). The experimental conditions employed were as described in ref. 36. Briefly, 4 pmol of each primer were used for the exponential amplification of the 749–956 region of 0.4 ng of the plasmid template DNA. The resulting PCR product was detected by agarose gel electrophoresis and isolated and purified using the Bio 101 GENECLEAN II Kit (National Diagnostics) following the manufacturer’s standard protocol. Drug-DNA incubations were carried out in TEOA buffer for 5 h at 37°C, in a total volume of 50 µL, using 10 µL of the purified probe per reaction, enough to yield an activity of at least 200 counts/s. Following precipitation and lyophilization, dried DNA pellets were heated at 90°C for 30 min in a total volume of 100 µL of sodium citrate buffer [1.5 mmol/L sodium citrate, 15 mmol/L NaCl (pH, 7.2)]. Samples were finally resuspended in 5 µL formamide loading buffer and subjected to electrophoresis.

**Studies in Saccharomyces cerevisiae**

**Yeast Strains and Cell Culture.** The haploid S. cerevisiae strains DBY747 (MATa his3-A1 leu2-3, 112 trp1-289 ura3-52), the rad4::hisG-URA3-hisG (WXY9394) and rad18::TRP1 (WXY9326) strains were kind gifts of Dr. Wei Xiao (University of Saskatchewan, Saskatoon, Saskatchewan, Canada; ref. 37). The rad4 rad18 strain (PJM35) was made by the disruption of the rad4 gene of strain WXY9326 with the KanMX4 cassette as described (38, 39). The maq1::hisG-URA3-hisG strain (JC8901; ref. 40) was a kind gift by Dr. L. Samson (Massachusetts Institute of Technology, Cambridge, MA), whereas the other BER mutant strains were obtained from the Saccharomyces Genome Deletion Project and were in the BY4741 background (MATa his3A1 leu2-3, 112 trp1-289 ura3-52, M recomA1 ura3-3A0), where the genes are disrupted with KanMX4.

**Survival Analysis.** Survival analysis was done as described in ref. 39.

**Measurement of Sequence Selectivity of Interaction in Intact Cells Using the Single-Strand Ligation PCR Assay.** The PCR-based technique, employed to detect induction of damage and follow the repair of the DNA adducts at the nucleotide level in cells, has been previously described for use with mammalian cells (41, 42). Its application on yeast cells has also been reported (32, 39). The analysis on the transcribed strand was done using 0.04 µg of digested genomic DNA with primers, experimental conditions, and procedures as detailed in ref. 32. Briefly, a single colony from the appropriate yeast strain was grown overnight in 100 mL yeast extract peptone dextrose (YPD). Cells were counted, pelleted (500 g, 10 min), and resuspended in 60 mL PBS at a concentration of 3 × 107 cells/mL. About 50 mL of cells were treated with 10 µmol/L AS-I-145 for 3 h at 28°C in an orbital shaker, and 10 mL of cells were mock treated with drug solvent. After drug treatment, cells were washed twice with PBS; 10 mL of cells were removed for the time 0-h sample, and the remaining 40 mL was resuspended in YEPD medium and incubated at 28°C for 2, 4, 6, or 8 h. The mock-treated samples were also incubated for 8 h. After this posttreatment incubation, the cells were centrifuged at 500 × g for 10 min at 20°C. Genomic DNA was purified using a Nucleo Mini Kit for yeast DNA extraction (Nucleon Biosciences). The genomic DNA was cut with 10 units of Rsal (NEB) overnight. The DNA was precipitated, and the DNA concentration was determined fluorimetrically.

First-round primer extension was carried out in a volume of 40 µL using 0.04 µg digested DNA. The PCR was carried out using 0.6 pmol of 5-biotinylated primer 2.5B and the reaction mixture composed of 20 mmol/L (NH4)2SO4, 75 mmol/L Tris-HCl (pH, 9.0), 0.01% (w/v) Tween 20, 4 mmol/L MgCl2, 0.2% gelatin, 250 µmol/L each dNTP, and 1 unit Taq polymerase. The DNA was initially denatured at 94°C for 4 min and then subjected to 30 cycles of 94°C, 1 min, 51°C, 1 min, 72°C, 1 min + 1 s extension per cycle on a PT-100 thermal cycler with a hot bonnet (MJ Research). The mixture was subjected to a final extension at 72°C for 5 min and then cooled to 4°C. To capture and purify the products of biotinylated primer extension, 10 µL of 5× washing and binding buffer [WBB; 5 mmol/L Tris-HCl (pH, 7.5), 1 mmol/L EDTA, 1 mol/L NaCl] was added to the PCR mixture, followed by 5 µL washed streptavidin M-280 Dynabeads (Dynal). The suspension was incubated for 30 min at 37°C with occasional agitation. The beads were sedimented in a magnetic rack and washed thrice with 200 µL 10 mmol/L triethanolamine (TE) (pH, 7.6). The beads were resuspended in 10 µL ligation mixture containing 10× T4 RNA ligase buffer [including 1 mmol/L hexamethionol(III) chloride], 50% polyethylene glycol (PEG) 8000, 20 pmol ligation oligonucleotide, and...
20 units of T4 RNA ligase and ligated overnight at 22°C. The ligation oligonucleotide was supplied with a 5' phosphate, essential for ligation, and a 3' terminal amine, which blocks its self-ligation. After ligation, the beads were washed thrice with 200 μL TE (pH, 7.6) and resuspended in 40 μL double-distilled water (ddH2O) for the second-round PCR. The second-round PCR mixture, in a volume of 100 μL, contained 10 pmol of each primer 2.5C and ligation primer that was complementary to the ligated oligonucleotide. The buffer composition was as for the first round PCR except that 2.5 units of Taq polymerase were used. The cycling conditions were an initial denaturation at 94°C for 4 min, then 18 cycles of 94°C, 1 min, 58°C, 1 min, 72°C 1 min + 1 s extension per cycle. The mixture was finally incubated at 72°C for 5 min and cooled to 4°C for 1 h. The third-round labeling reaction was carried out by adding 10 μL of 32p 5' end-labeled primer 2.5D (5 pmol) and 1 unit Taq polymerase in PCR reaction buffer. The mixture was subjected to four further cycles of 94°C, 1 min, 61°C, 1 min, and 72°C, 1 min, 72°C, 5 min, and 4°C, 1 h. The beads were removed by spinning briefly and rinsed with 100 μL ddH2O. The supernatant was removed and precipitated with ethanol. Samples were finally resuspended in 5 μL formamide loading buffer.

For analysis on the non-transcribed strand, the same amount of genomic DNA (0.04 μg) was cut with 10 units of DdeI (NEB) overnight. The same experimental conditions were applied with the oligonucleotide primers: 2.4B 5'-biotin-TATAAGAGTTTATAGTGGTGAAG-3'; 2.4C, 5'-AGTGGTGAAAGATAGTGG-3'; 2.4D, 5'-GATAGTGGGACTTGTGACTGGT-3' employed at the corresponding steps of the procedure. The oligonucleotide primer sets for use in these studies were synthesized by MWG (Ebersberg, Germany).

Samples dissolved in formamide loading dye were denatured at 95°C for 3 min, cooled on ice, and electro-phoresed at 1,500 V for 3 h in a 50-cm × 21-cm × 0.4-mm 6% polyacrylamide sequencing gel (SequaGel 6; National Diagnostics). The gels were dried and autoradiographed (Kodak Hyper film, Amersham).

Studies in Chinese Hamster Ovary Cells

Cell Lines and Culture Conditions. The AA8, UV23, UV42, UV61, and UV96 cell lines were obtained from Dr. M. Stefanini (Istituto di Genetica Biochimica et Evoluzionistica, Pavia, Italy), and UV135 was purchased from the American Type Culture Collection (ATCC number CRL-1867). All cell lines were grown in T80-cm² tissue culture flasks (Life Technologies, Pavia, Italy) and supplemented with 2 mmol/L glutamine (Sigma) and 10% fetal calf serum (FCS; Autogenbioclear). The latter had been heat inactivated by a 30-min treatment at 65°C. Cells were grown at 37°C in a 5% CO₂ humidified incubator and harvested using trypsin-EDTA 1x solution (Autogenbioclear).

Growth Inhibition Assay. Cytotoxicity was assessed using the sulforhodamine B (SRB) assay (43). Experimental conditions and procedures are as described in ref. 44. Dose-response curves were plotted, and the IC₅₀ values, i.e., the drug concentration required to produce 50% of the absorbance of the untreated control, were determined. When comparing parental and mutant cell lines, the relative sensitivity was determined by the following equation:

Relative sensitivity = IC₅₀ value in parent cell line / IC₅₀ value in the mutant cell line

In vivo Studies

NCI Standard Hollow Fiber Assay. This initial assessment of in vivo activity using cells transferred to polyvinylidenefluoride (PVDF) hollow fibers and grown in the intraperitoneal and subcutaneous compartments of mice was conducted by the NCI as described previously by Hollingshead et al. (45).

Human Tumor Xenograft Model. Human breast cancer MDA-MB-435 (1 × 10⁶ cells) was implanted s.c. in athymic nude mice. Palpable tumors appeared within 12 days and measured ~230 mm³ as estimated by two-dimensional caliper measurements and using the formula for an ellipsoid. Before chemotherapy, mice were pair matched by tumor volume into groups of seven animals, and doses of AS-I-145 were given p.o. or i.v. on days 1, 5, and 9. Tumor volumes were periodically measured using calipers.

Results

DNA Sequence-Selective Alkylation by AS-I-145

Figure 2A shows the sites of covalent binding of AS-I-145 on one strand of a fragment of pUC18 DNA, as identified by the Taq polymerase stop assay. The predominant site of polymerase stop is the cluster of five adenines, 865-AAAAA-3' (8). This is also a major binding site for adozelesin, which shows an approximately 10-fold greater reactivity. Another alkylation site of adozelesin on this fragment is the 5'-TTTA-3′ site, which is only poorly reacted by AS-I-145, with stop sites only detectable at the highest concentration of 10 μmol/L.

A thermally induced strand cleavage assay was subsequently done to confirm the binding in the minor groove of DNA and probe the covalent modifications at purine-N3 positions. The fragment investigated in this assay includes the region of the strand employed as a template for drug modification and linear amplification in the Taq polymerase stop study, allowing for direct comparison between the two assays. The autoradiograph of the thermal cleavage gel is shown in Fig. 2B. The gel reveals the same cluster of adenines as the predominant site of drug-DNA interaction, with the 3' adenine within the A₅ (869–865) being the most reactive to AS-I-145. A cleavage product corresponding to the A (843) is only evident for adozelesin, confirming the poor affinity of AS-I-145 for this site. Additional sites of alkylation at the top portion of the autoradiograph of Fig. 2B include the AT mixed sequences.
at positions $76^\prime$-ATT-$3^\prime$ and $77^\prime$-ATA-$3^\prime$ (the $3'$-alkylated adenine is underlined). Both sites are alkylated by adozelesin, but only the latter is alkylated weakly by the achiral agent. In all instances, the alkylated bases detected by the thermally induced cleavage assay were adenines flanked by at least two $5$' A or T bases, with AS-I-145 being more sequence discriminating that adozelesin. These observations suggest that despite the lack of a chiral center, AS-I-145 retains the intrinsic sequence selectivity of the chiral pharmacophores of CC-1065 and the duocarmycins, sharing a high affinity for contiguous adenines, and to a lesser extent, AT mixed sites. The proposed model for activation and adenine-N3 alkylation by AS-I-145 is shown in Fig. 2C.

**Repair of AS-I-145 Adducts in *S. cerevisiae***

To define the repair pathways acting on the adenine-N3 adducts induced by AS-I-145, we compared the sensitivity of a repair-proficient *S. cerevisiae* parent strain (DBY747) to isogenic disruptants defective in DNA repair pathways. A rad4 mutant that is completely deficient in NER exhibited
increased sensitivity over the wild type (Fig. 3A). In contrast, the BER mutant mag1, defective in the activity of 3-methyladenine DNA glycosylase, an enzyme known to repair simple guanine-N7 and adenine-N3 monokylations, displayed an indistinguishable sensitivity to the parental strain (Fig. 3A). The epistatic relationship between the two excision pathways was investigated with the examination of a rad4 mag1 double disruptant. The double mutant was as sensitive as the most sensitive single NER mutant (Fig. 3A). This suggests that the observed sensitivity of the rad4 mag1 cells is predominantly dependent on the NER defect. Isogenic strains disrupted for several other BER activities were also screened for AS-I-145 sensitivity, including the ogg1 (8-oxoguanine/formamidopyrimidine glycosylase), the DNA glycosylase/AP lyases ntg1 (8-oxoguanine glycosylase/lyase), ntg2 (related to Escherichia coli endonuclease III), and ung1 (uracil-DNA glycosylase). None of these BER defects conferred increased sensitivity to AS-I-145 over the isogenic parent (data not shown), implying that BER does not play a major role in the elimination of the AS-I-145 adenine-N3 adducts.

Marked sensitivity to AS-I-145 was also observed in a rad18 disruptive strain as shown in Fig. 3B. Rad18 is one of the pivotal genes in the PRR pathway, forming a stable heterodimer in vivo with Rad6. Comparison of the sensitivity of a rad4 rad18 double disruptant with the rad4 and rad18 single mutants indicated that RAD4 and RAD18 are not epistatic for the AS-I-145 damage response because the double mutant was more sensitive than either of the single mutants (Fig. 3B). This complex pattern of epistasis between RAD4 and RAD18 led us to investigate whether the RAD18 gene product influences the repair rates of individual lesions.

Single-strand ligation PCR (sslig-PCR) was employed to follow the induction and repair of the AS-I-145 adducts along the active yeast MFA2 gene, in a region of high AT content. The repair capacity of the rad4 and rad18 mutants compared with their isogenic repair-proficient parent was assessed using primers specific for the transcribed strand (Fig. 4A). Several prominent sites of adenine adduct formation are detected by sslig-PCR, located within runs of contiguous adenines and some mixed AT sequences. The occurrence of the adducts along this region of the gene was identical among the different strains studied. Lane U is the untreated control, 0 is DNA extracted from AS-I-145 cells immediately after the drug treatment, whereas 8 is DNA from cells allowed to repair in YEPD for 8 h before extraction. It is clear that the AS-I-145 adducts are largely eliminated in the repair-proficient parental strain within the 8 h of posttreatment recovery. In contrast, rad4 cells fail to remove any of the adducts, suggesting that damage removal observed in the wild type involved NER. The Rad18 defective strain also failed to eliminate any of the DNA adducts over 8 h. The high level of sensitivity observed in the rad18 cells could therefore be attributed to their inability to eliminate the adducts, suggesting a contribution of the RAD6 pathway in the excision repair of this type of sequence-specific minor groove adducts.

The repair capacity of the parental DBY747 strain was also assessed on the non-transcribed strand of the MFA2 gene (Fig. 4B). Because little repair is evident after the 8 h of posttreatment incubation, it is deduced that the NER component of the repair observed at MFA2 is transcription coupled because removal of adducts occurs preferentially on this strand.

**Repair of AS-I-145 Adducts in Mammalian Cells**

To further establish the critical role of NER in the repair of the AS-I-145 adducts, a panel of Chinese hamster ovary (CHO) cell lines, with specific defects in different components of NER, was employed. The sensitivity of the CHO cells to AS-I-145 was assessed using the SRB growth incubation assay after a 1-h exposure to increasing concentrations of the agent. Figure 5 shows the sensitivity of the wild-type (AA8) and the NER-defective repair mutants. Increased sensitivity was observed in all six of the mutants tested, ranging from 4- to 7.9-fold (Table 1). These data confirm the role of NER in mammalian cells in the repair of the AS-I-145 adducts. In particular, the increased sensitivity of the UV61 cells defective in CSB suggests that survival depends on transcription-coupled NER, as observed in yeast.

**In vivo Antitumor Activity of AS-I-145**

Following the demonstration of differential activity in the NCI 60 cell line in vitro screen (31), AS-I-145 was
tested in the standard NCI hollow fiber assay against NCI-H522, NCI-H23, LOX IMVI, UACC-62, OVCAR-3, OVCAR-5, MDA-MB-435, MDA-MB-231, CoLo-205, SW-620, U-251, and SF-295. Treatment with AS-I-145 i.p. at doses of 12 and 18 mg/kg given daily for 4 days produced an IP score of 46 and a SC score of 8, achieving a composite score of 54, with evidence of cell killing. AS-I-145 is therefore highly active in this assay resulting in further in vivo evaluation using human tumor xenografts.

Activity was previously reported against the murine B16 melanoma and the human ovarian tumor OVCAR-3 xenograft when the agent is given i.p. (31). Activity is also evident when AS-I-145 is given by the i.v. or p.o. routes. For example, Fig. 6 shows the result of an efficacy experiment against the human breast cancer MDA-MB-435 subcutaneous xenograft. Clear inhibition of tumor growth was evident at all the oral doses given (70–280 mg/kg), and no weight loss was observed at any dose. Activity was also seen when the drug was given at 10 mg/kg i.v. Antitumor activity was also observed against the human SF-295 glioma, OVCAR-3 ovarian and NCI-H522 lung cancers when given p.o. (data not shown).

Discussion

We have previously reported the design, synthesis, and biological evaluation of several classes of achiral duocarmycin analogues, including seco-hydroxy-CI analogues (25), a seco-amino-CI-TMI agent (26), seco-duocarmycin SA analogues (46), and a seco-hydroxy-CBI-TMI, along with

![Figure 4. A, repair of AS-I-145 adducts on the transcribed strand (TS) of MFA2. Exponentially growing DBY747, rad4, and rad18 yeast cells were treated with 10 μmol/L AS-I-145 for 3 h at 30 °C. Cells were harvested, and DNA was extracted immediately (0-h lane) or allowed to repair in YEPD for 8 h before DNA extraction (lane 8). U, untreated control. sslig-PCR was done as described in Materials and Methods. Arrows, location of the major adducts; their sequence context and position in relation to the start of the coding region (+1) are indicated.](http://mct.aacrjournals.org/content/mct/6/10/2714.full)

**Figure 4. A**, repair of AS-I-145 adducts on the transcribed strand (TS) of MFA2. Exponentially growing DBY474, rad4, and rad18 yeast cells were treated with 10 μmol/L AS-I-145 for 3 h at 30 °C. Cells were harvested, and DNA was extracted immediately (0-h lane) or allowed to repair in YEPD for 8 h before DNA extraction. sslig-PCR was done as described in Materials and Methods. Arrows, location of the major adducts; their sequence context and position in relation to the start of the coding region (+1) are indicated. **B**, repair of AS-I-145 adducts on the non-transcribed strand (NTS) of MFA2. Exponentially growing DBY747 cells were treated with 10 μmol/L of AS-I-145 for 3 h at 30 °C. Cells were harvested, and DNA was extracted immediately (0-h lane), or cells were resuspended in YEPD and allowed to repair for a further 8 h before DNA extraction. sslig-PCR was done as described in Materials and Methods. Arrows, location of the major adducts; their sequence context and position in relation to the start of the coding region (+328) are indicated. The sequence of the portion of the gels presented here stretches from positions 460 to 230. The NTS analysis depicted in the autoradiograph also encompasses sequences downstream of the transcription termination site (+328) because no prominent AS-I-145 adducts were detectable further upstream and along the region examined in the repair analysis of the TS.
various seco-amino-CBI analogues (31). The latter class of achiral agents is exemplified by AS-I-145, which bears the TMI group as the DNA binding domain. The full complexity of the natural products is not required for high potency (47), and a number of distinct advantages of incorporating the CBI pharmacophore over CI and CPI have been established from early structure-activity relationship studies of structurally altered alkylation subunits in chiral analogues (27–30).

The alkylation properties of AS-I-145 were determined on plasmid DNA. Sites of covalent modification were revealed using the Taq polymerase stop assay, and purine-N3 alkylation in the minor groove was probed using the thermally induced cleavage assay. The objective of the alkylation studies was to determine whether the achiral pharmacophore can still be an effective alkylator and if this modification would have any effect on the alkylation efficiency/sequence selectivity reported for the natural lead compounds. Recent studies have highlighted the validity of such studies on naked DNA and their importance as a valuable tool and a good approximation of the expected intracellular behavior and in vivo binding of this class of compounds (48). More specifically, the DNA alkylation properties of both enantiomers of DUMSA and yatakemycin were determined on protein-free DNA and on the same sequence bound in nucleosome core particle (NCP), modeling the state of DNA in eukaryotic cells. The relative efficiencies of the agents to alkylate DNA and their sequence selectivity were shown to remain unaltered in both instances.

In the present study, AS-I-145 was shown to efficiently alkylate AT-rich sequences with a decreased potency, but increased selectivity, compared with adozelesin. Similar to the loss of HCl in previously reported seco-CI (49–51) and seco-CBI (30, 52) chiral analogues in biological media, AS-I-145 is believed to lose HCl to produce the putative cyclopropane-containing alkylating agent leading to the observed alkylation. The sequence selectivity observed with short fragments of DNA was preserved in the intracellular environment of yeast as revealed by the occurrence of adduct formation in AT-rich sequences along the MFA2 gene of S. cerevisiae. In addition to AT sequence specificity, the distribution of adducts in domains of the genome, referred to as region specificity, may also contribute to the biological outcome (53).

The sequence-selective adenine-N3 adducts induced by AS-I-145 were shown to be substrates for NER. CC-1065 adducts have been shown to be substrates for bacterial and human NER (54–56). NER is proposed to be the prominent excision pathway involved in the repair of the AS-I-145 adducts because of all the BER glycosylases tested, none was found to be more sensitive compared with the wild type. These results are in agreement with previous findings on CC-1065/duocarmycin analogues, suggesting that BER is not a crucial cellular response to alkylation by this class of compound. It has previously been shown that the 3-methyladenine-DNA-glycosylase does not play a major role in the repair of the adenine adducts formed by CC-1065 because the introduction of the bacterial gene (tag) encoding this enzyme in different murine cells did not change the sensitivity of the cells to the drug (57). Additionally, no correlation between the levels of the glycosylase and the sensitivity to CC-1065 in human cancer cell lines was found in another study (58). An adenosine-N3 adduct formed by adozelesin was also shown to be a substrate for the NER action of the E. coli endonuclease UvrABC, but the E. coli 3-methyladenine glycosylase failed to excise the adenosine lesions (59).

The lack of Rad18 not only increased the sensitivity of the yeast cells to AS-I-145, but impaired their ability to eliminate the adducts by NER as shown on the region of the MFA2 gene studied by sslig-PCR. Rad18, a DNA binding protein that also possesses ubiquitin ligase and ATPase activities is one of the pivotal genes in the PRR pathway, forming a stable heterodimer in vivo with Rad6, a ubiquitin-conjugating enzyme (60–62). PRR is an umbrella term for a number of pathways required for the tolerance and repair of lesions encountered by replication forks,

### Table 1. The CHO cell lines used in this study, their gene defect, and the IC_{50} values of AS-I-145 determined using the SRB assay

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Gene defect</th>
<th>AS-I-145 IC_{50} (nmol/L)</th>
<th>Sensitivity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA8</td>
<td></td>
<td>190</td>
<td></td>
</tr>
<tr>
<td>UV23</td>
<td>XPB</td>
<td>28.5 ± 0.7</td>
<td>6.6</td>
</tr>
<tr>
<td>UV42</td>
<td>XPD</td>
<td>36.0 ± 5.6</td>
<td>5.3</td>
</tr>
<tr>
<td>UV47</td>
<td>XPF</td>
<td>26.0 ± 7.0</td>
<td>7.3</td>
</tr>
<tr>
<td>UV61</td>
<td>CSB</td>
<td>47.0 ± 2.8</td>
<td>4.04</td>
</tr>
<tr>
<td>UV96</td>
<td>ERCC1</td>
<td>24.0 ± 11.3</td>
<td>7.9</td>
</tr>
<tr>
<td>UV135</td>
<td>XPG</td>
<td>34.6 ± 3.2</td>
<td>5.5</td>
</tr>
</tbody>
</table>

NOTE: The sensitivity ratio is the fold increase in sensitivity of the mutant cell lines compared with the parental cell line AA8.
either aiding repair or tolerance at the stalled fork, or providing a means of stabilizing stalled-fork intermediates (including gaps) that can be processed at the end of bulk DNA replication. Rad6 and Rad18 play a crucial part in regulating all known sub-pathways that comprise the error-free and the error-prone modes of PRR (63). The requirement of functional Rad18, and the related Rad5 factor, for the excision by NER of the same type of adducts, has previously been shown for the related compound TH-III-151 (39). Rad18 cells were completely deficient in the excision (by a yet unidentified mechanism) of another type of highly sequence-specific minor groove adducts produced by a benzoic acid mustard tethering three pyrrole groups (32). In that case, it was postulated that Rad18 may be involved in the recognition of poorly displayed minor groove adducts, acting as a damage sensor and recruiting repair factors required for their elimination. The data demonstrating the transcription-coupled nature of the NER occurring at AS-I-145 lesions and the other minor groove adducts described above, taken together with a requirement for Rad18, suggest that these adducts are only efficiently detected following collision with a DNA (during replication) or RNA (during transcription) polymerase. This could explain the dependence on transcription and a Rad18-dependent pathway for efficient repair. Indeed, previous biochemical studies (56) using human cell extracts showed that CC-1065 adducts were relatively poorly incised in vitro when compared with major groove lesions such as those produced by acetylaminofluorene and UV light. Two possible explanations presented for this were the relatively low level of DNA structural distortion induced by CC-1065 adducts and/or thermodynamic stabilization of the double helix by these adducts (64).

Studies in mammalian cells also revealed the importance of transcription-coupled NER in the repair of AS-I-145–induced damage. Previously, UV23, UV42, UV47, and UV96 cells displayed increased sensitivity (2-fold) to the minor groove alkylation agents tallimustine and CC-1065 in a colony formation assay (65). In the present study, a 4-fold enhanced activity of AS-I-145 was observed in the UV61 cell line, defective in CSB, the Cockayne syndrome–related factor involved in the transcriptional coupling of NER. Higher efficiency of CC-1065 adduct removal and significantly faster repair in the transcribed strand than in the non-transcribed strand of the dihydrofolate reductase (DHFR) gene was previously observed in CHO cells. This preferential repair was attributed to transcription-coupled repair of active genes (66).

The natural antitumor compound illudin S and its semisynthetic derivative irofulven both belonging to the family of illudins are the only other reported examples in the literature, with transcription-coupled NER and Rad18 being determinants of chemosensitivity (67, 68). However, the exact nature of their interaction with DNA has not been elucidated. Another similarity between AS-I-145 and the illudins is the preferential activity of all these agents against solid tumors over leukemias, as determined in the NCI in vitro screen. However, this does not apply to all members of the broader class of the minor groove binding agents. Ecteinascidin 743 (ET-743, NSC648766, Yondelis), for example, a carbinolamine-containing naturally occurring antitumor antibiotic that binds in the minor groove of DNA and selectively alkylates the N2-position of guanine, exhibits a distinctly different pattern of NER sensitivity (69, 70). Defective mammalian cell lines were found to be more resistant rather than more sensitive to this agent, compared with their isogenic parent cell lines. This NER-mediated cytotoxicity of ET-743 was additionally proposed to be specifically dependent on the transcription-coupled pathway of NER (70).

We have previously reported, as part of preliminary studies of AS-I-145, the ability of the agent to inhibit the growth of murine B16-F0 melanoma in C57BL/6 mice and the OVCA-3 human ovarian cancer xenograft in severe combined immunodeficiency mice when given i.p. (31). In this report, we show antitumor activity against the human breast MDA-MB-435 xenograft when given either i.v. or p.o. The oral bioavailability of AS-I-145 is, however, low. Nevertheless, it is evident that achiral molecules of this type provide a new platform for the development of active antitumor agents that can be exploited in the future.

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References


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DNA sequence–selective adenine alkylation, mechanism of adduct repair, and in vivo antitumor activity of the novel achiral seco-amino-cyclopropylbenz[e]indolone analogue of duocarmycin AS-I-145

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