Chemistry & Biology 13, 485–492, May 2006 DOI 10.1016/j.chembiol.2006.02.015

Cellular Effects Induced by the Antitumor Agent Azinomycin B

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Summary

Studies on the mechanism of action of the antitumor agent azinomycin B in vitro suggest that the drug elicits its lethal effects by the formation of interstrand crosslinks within the major groove of DNA. Here, we demonstrate the biological effects of the drug in vivo. Fluorescence imaging revealed localization of azinomycin B in the nuclear region of yeast. Moreover, experiments with oligonucleotide microarrays examined the effects of the drug across the yeast transcriptome. The results demonstrated a robust DNA damage response that supports the proposed role of the drug as a covalent DNA modifying agent. RT-PCR analysis validated the gene changes, and flow cytometry of azinomycin-treated yeast cells demonstrated a phenotypic S phase shift consistent with transcriptional effects.

Introduction

Azinomycins A and B (Figure 1) are naturally occurring antitumor agents that are among a small set of molecules that interact with DNA in the major groove (for a review of agents that covalently modify DNA, see [1]). Azinomycin B was originally isolated from *Streptomyces sahachiroi* in 1954 as carzinophilin A [2] and, along with azinomycin A, was subsequently reisolated from another *Streptomyces* strain, *S. griseofuscus*, in 1986 [3, 4]. The total synthesis of azinomycin A was achieved in 2001 [5], and numerous reports on synthetic approaches to these natural products have been reported [6, 7].

The seminal discovery that azinomycin B interacts with DNA, forming interstrand crosslinks without prior activation, was achieved by Lown and Majumdar [8]. Since this initial demonstration, a substantial number of in vitro studies have revealed both the regioselectivity and apparent sequence selectivity of the compound [9–13]. Azinomycin B interacts with the duplex DNA sequence 5′-d(PuNPy)-3′ [9, 11] to form covalent interstrand crosslinks via the electrophilic C10 and C21 carbons of azinomycin and the N7 positions of suitably disposed purine bases [9, 13]. Kinetic assays together with synthetic work further suggest that the monoalkylated product is formed first through reaction with the aziridine, followed by crosslinking with the epoxide [9]. The highest levels of crosslinked product are attained with the DNA recognition sequence 5′-d(GGC·CCG)-3′ [9].

Viscometry, fluorescence contact energy transfer, and DNA unwinding experiments to probe the role of the azinomycin naphthoate point to a nonintercalative binding mode for this group [9]. This is in contrast to the neocarzinostatin naphthoate, which, despite structural similarity with the azinomycin naphthoate, has been demonstrated to exhibit an intercalative binding mode [14]. In support of these results, computer modeling studies point to a nonintercalative binding mode for this molecule [15, 16]. These results, however, still leave unanswered the question of whether DNA is the relevant cellular target of this agent.

Despite extensive work to characterize the interaction of azinomycin B with its DNA receptor in vitro, data on the in vivo actions of the molecule are limited to the initial description of its cytotoxicity and an early phase clinical investigation [17, 18]. In this report, the natural products were shown to exhibit potent cytotoxic activity (IC_{50} = 0.07 μg/ml [Figure 1A] and 0.11 μg/ml [Figure 1B]) against the leukemia cell line L5178Y. Azinomycin B (Figure 1B) provided a 193% increased life span (ILS) at 32 μg/kg/day (3/7 survivors) against an IP-implanted P388 leukemia mouse model. In the same system, mitomycin C exhibited a 204% ILS, but at 1 mg/kg/day. A preliminary clinical investigation with the compound (Figure 1B) gave favorable results in 36 cases of malignant neoplasms. Remarkable efficacy was observed in a case of squamous cell carcinoma (a form of skin cancer). Local administration of the agent led to rapid reduction of the ulcer surface and eventual disappearance of the tumor cells [18].

Herein, we provide the first evidence for in vivo DNA damage by azinomycin B by using cellular localization studies and transcriptional profiling experiments across the yeast genome. Our results suggest that the well-established DNA alkylation and crosslinking exhibited by this agent in vitro are likely biologically relevant to lesions in vivo.

Results and Discussion

In the original 1987 report, azinomycin B was found to have no biological effect against yeast [17]. We have examined azinomycin B against cultures of *Saccharomyces cerevisiae* and observed cytotoxic activity at moderate concentrations (ED_{50} = 10 μg/ml). In this report, we have used yeast as a model organism to establish the cellular origin of cytotoxicity exhibited by this agent.

Cellular Localization of Azinomycin B

In efforts to substantiate the purported biological role of azinomycin B as a DNA crosslinking agent, we initially examined the localization pattern of the agent in yeast by using fluorescence microscopy. In earlier work, Coleman and coworkers reported the following UV/vis

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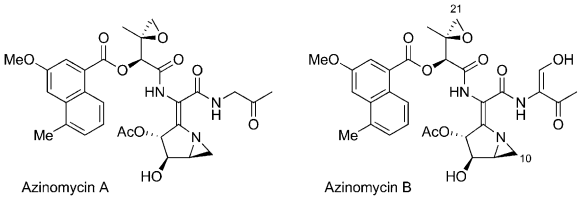

Figure 1. Structures of Azinomycins A and B

spectrum for the azinomycin naphthoate: λ_{max} (MeOH/H_2O, 1:1) 240 (ε 34,000), 298 (ε 6,300), 338 nm (ε 7,400). They also described fluorescence contact energy transfer experiments in which emission by the naphthoate was observed at 435 nm [9]. This fluorescence range is comparable to that exhibited by the fluorescent stain DAPI, which has excitation and emission wavelengths of 345 nm and 455 nm, respectively.

At 1000× amplification, yeast treated with azinomycin B exhibited localized binding and fluorescence (Figure 2). *Saccharomyces cerevisiae* cells were treated with azinomycin B (100 μg/ml and a blast treatment of 4000 μg/ml; Figures 2A and 2B) or the naphthoate core of azinomycin (DNA nonbinder; 500 μg/ml; Figure 2D), and they were compared to ethanol-fixed yeast cells (Figure 2C) and fixed cells stained with the DNA intercalator propidium iodide (1 ng/ml; Figure 2E). After incubation for 4 hr, cells were centrifuged and resuspended in phosphate-buffered saline. Cells were viewed with a Zeiss fluorescence microscope, and the images were captured digitally, under constant image capture conditions.

The pattern of fluorescence exhibited by azinomycin B paralleled that exhibited by propidium iodide, in which staining of the nuclear region of the cell was observed (i.e., the area of the cell that surrounds the central body or vacuole storage organelle of yeast). In contrast, control cells treated with ethanol showed diffuse background fluorescence (Figure 2C), as did yeast cells treated with the azinomycin naphthoate (a DNA nonbinder; Figure 2D). These results provide evidence that azinomycin B is concentrated in the cell nucleus, which would be expected for an agent that interacts covalently with duplex DNA.

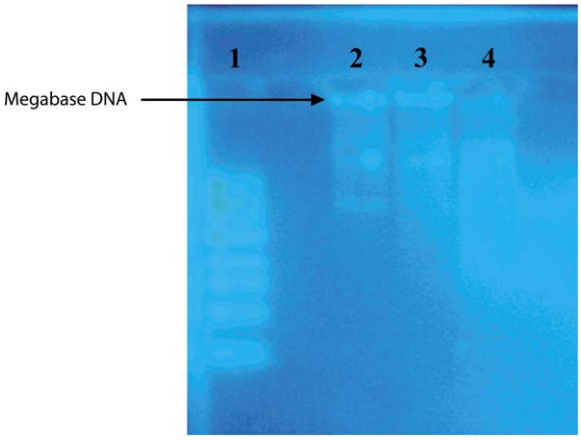

Figure 3. Effect of Azinomycin B on Genomic DNA, 1% Agarose Gel Visualized with SYBR Green

Yeast cells were cultured to 1.0 OD and treated with ethanol vehicle or azinomycin B for 12 hr. Lane 1, DNA ladder; lane 2, EtOH control; lane 3, azinomycin B (10 μl); lane 4, azinomycin B (100 μl).

DNA-Damaging Effect of Azinomycin B

The nuclear effects of the agent were further corroborated by the DNA damage that resulted with azinomycin exposure. Genomic DNA isolated from yeast treated with the natural product demonstrated increased shearing of the DNA as compared to yeast cells incubated with ethanol alone (Figure 3). Moreover, a dose-dependent response was observed.

Transcriptional Response and Cell Cycle Analysis

The effect of azinomycin B on the yeast transcriptome was examined by using Affymetrix oligonucleotide microarrays (Yeast S98 Array). Data were obtained at two concentrations of agent (Table 1). Drug treatment was carried out in duplicate at the ED_{50} concentration (10 μg/ml) and a 10-fold higher concentration (100 μg/ml) for 6 hr. The data in Table 1 are remarkably consistent with the proposed mode of action of azinomycin B as a DNA-damaging agent. Differential mRNA gene expression (≥2-fold) was observed in 47 transcripts out of ~6200 genes that comprise the yeast genome. Approximately 57% of those differentially expressed transcripts

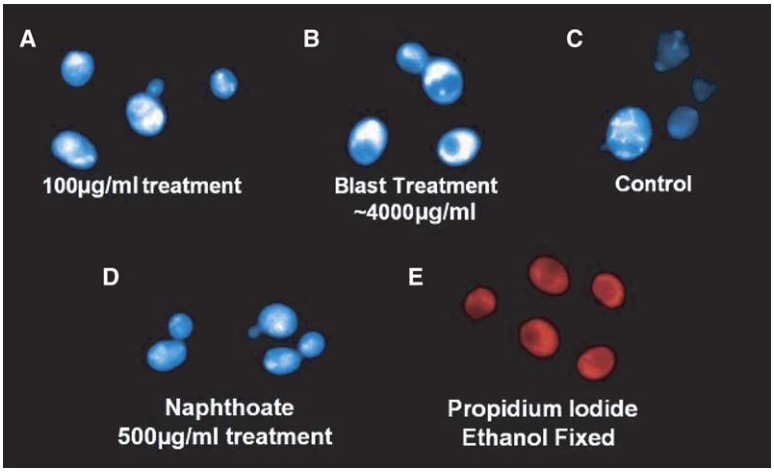

Figure 2. Cellular Localization of Azinomycin B

(A) Yeast treated with azinomycin B for 6 hr; 100 μg/ml.
(B) Yeast treated with azinomycin B for 6 hr; ~4000 μg/ml.
(C) Yeast treated with ethanol for 6 hr; control.
(D) Yeast treated with naphthoate (non-DNA binder); ~500 μg/ml.
(E) Yeast fixed with ethanol and stained with propidium iodide.

Table 1. Effect of Azinomycin B on Gene Expression in Yeast

Upregulated Probe Set	Gene Description	Fold Change 10 μg/ml	Fold Change 100 μg/ml
Nuclear/DNA Damage			
4045_s_at	YIL066C ribonucleotide reductase large subunit	2.5	—
5643_at	YER070W ribonucleotide reductase	—	3
5626_at	YER095W RecA homolog, RAD 51, involved in recombinational repair	—	3
5970_at	YDR501W, PLM2 plasmid maintenance	2.6	9
3262_at	YGLCTAU3 Ty4 LTR	—	14.6
3200_I_at	YHLWDELTA2 Ty1 LTR	—	23.7
10222_at	YLR103 chromosomal DNA replication protein	—	6.3
7352_at	YBL009W, strong similarity to DNA damage-responsive Alk1 p	—	4.1
3058_s_at	YHR015W DNA helicase and DNA-dependent ATPase involved in DNA repair	—	3.2
8639_at	YOL104C in meiotic chromosome segragation, might stabililize homologous DNA interactions at telomeres	—	4.3
6293_at	YDR191W, homolog of SIR2	—	9.08
Cell Signaling/Cell Cycle			
9293_at	YML058w-a identified by SAGE, Hug-1, involved in cell cycle checkpoint and arrest	16	73
5108_at	YGL089C α mating factor	2.1	5.7
8028_at	YPL267W weak similarity to *C. elegans* transcription factor unc-86	—	4.18
6021_f_at	YDR461W a-factor mating pheromone precursor	—	10.3
6585_at	YDL101C protein kinase	—	3.7
10164_at	YLR183C similarity to YDR501w, transcription factor induced in G1, bind to promoter in the cell cycle	—	21.6
Ribosomal Proteins			
7190_I_at	YBR189W ribosomal protein S9B (S13)(rp21)(YS11)	—	8.6
7191_f_at	YBR189W ribosomal protein S9B (S13)(rp21)(YS11)	—	7.1
6069_I_at	YDR418W ribosomal protein L12B (L15B)(YL23)	—	3.3
5076_f_at	YGL076C ribosomal protein L7A (L6A)(rp11)(YL8)	—	4.1
11237 _at	YJL189W ribosomal protein L39 (L46)(YL40)	—	4.7
11235_at	YJL191W ribosomal protein S14B (rp59B)	—	6.4
10068_I_at	YLR264W ribosomal protein S28B (S33B)(YS27)	—	8.3
9497_I_at	YMR143W ribosomal protein S16A (rp61R)	—	4.5
9006_s_at	YNL162W ribosomal protein L42A (YL27)(141A)	—	4.5
Unclassified Genes			
7792_at	YPL051W strong similarity to ADP-ribosylation factors	—	3.8
7697 _I_at	YPR077C questionable ORF, gene function unknown	—	4.3
9075_at	YNL227C similarity to dnaJ-like proteins, possible cochaperone	—	6.6
4854_at	YGR153W hypothetical protein, uncharacterized	—	4.4
49R1 at	YGR035C hypothetical protein, potential Cdc28p substrate	—	6.1
5365_at	YFR023W poly(A) binding protein, related to PES4 protein homolog	—	3.5

Downregulated Probe Set	Gene Description	Fold Change 10 μg/ml	Fold Change 100 μg/ml
Nuclear/DNA Damage			
3310_s_at	YERWDELTA18 Ty1 LTR	—	−5.8
4692_f_at	TY element transposition	—	−5.9
7513_f_at	TY element transposition	—	−4.7
8752_f_at	TY element transposition	—	−5.7
11323_at	YAL001C transcription factor tau (TFIIIC) subunit 138	−3.2	—
3248_s_at	YGR109W-B, DNA and RNA binding, RNA-dependent DNA replication, transferase activity	−3	—
3712_s_at	YNRCTY1-3 full-length Ty1, transposon	−3.9	—
3913_s_at	YARCTY1-1 full-length Ty1, transposon	−3.6	—
Cell Signaling/Cell Cycle			
2350 s at	YDR457W or Tom1, ubiquitin ligase, required for G2/M transition	−3.2	—
Transport			
4842_at	YGR142W involved in intracellular protein transport	−3.6	—
2642_s_at	YOR153w or PDR5, transporter, exhibits nucleoside triphosphatase activity	−3.9	—
2643_s_at	YOR153w or PDR5, transporter, exhibits nucleoside triphosphatase activity	−4	—
Mitochondrial			
10934_at	YJR095W protein related to mitochondrial carriers	—	−7.8
5679_at	YER024W similarity to carnitine O-acetyltransferase Yat1p	—	−4
4005_at	Cytochrome C oxidase subunit I	—	−7.2

were nuclear related, including genes involved in DNA repair, DNA maintenance, and cell cycle control as well as genes for transposons. The remaining genes included ribosomal proteins, mitochondrial genes, transporter proteins, and a few unclassified genes.

Among the nuclear effects, Ty elements were largely affected by azinomycin treatment. While the expression of YGLCtau3 Ty4 LTR and YHLWdelta2 Ty1 LTR was upregulated by a remarkable 15- and 24-fold, respectively, most transposition elements (a total of 7) were repressed 3- to 6-fold. LTR retrotransposons have been extensively studied in *Saccharomyces cerevisiae*, and five distinct families, designated Ty1, Ty2, Ty3, Ty4, and Ty5, have been identified in this organism [19]. The Ty elements transpose through an RNA intermediate by reverse transcription [20]. While differential expression of these genes could reflect a general cellular stress response, differential expression was not observed with other DNA-damaging agents, including the DNA-alkylating agent methyl methanesulfonate and the DNA-cleaving agent calicheamicin γ_1I [21]. Moreover, recent studies have shown that when DNA replication is compromised in yeast, Ty elements constitute a preferred site for double-strand DNA breaks, analogous to fragile sites observed in mammalian chromosomes [22].

Additionally, classification and correlation of the majority of the gene changes revealed activation of genes in the MEC1 checkpoint or sensory pathway, which is involved in DNA double-strand break repair (Figure 4). DUN1, a serine-threonine kinase required for DNA damage-induced transcription, plays a pivotal role in the MEC1 pathway and was enhanced in its expression by 4-fold. The cell cycle checkpoint protein induces G2/M arrest after DNA damage and controls postreplicative DNA repair [23]. In this regard, we observed downregulation of TOM1 (YDR457w), a ubiquitin ligase required for the G2/M transition [24, 25]. The yeast pheromones α-mating factor and a-mating factor were upregulated 6- and 10-fold, respectively, in expression. Both pheromones act by G1 phase synchronization of cell populations in preparation for mating [26]. The expression of MF(ALPHA)2 was upregulated 2-fold and 6-fold at 10 μg/ml and 100 μg/ml, respectively, and MFA1 was upregulated 10-fold. Cells respond to these pheromones during conjugation and cellular fusion, and they interact to induce cell cycle arrest [27, 28]. Tos4 (or TLR183c), a transcription factor that binds promoter regions of genes involved in pheromone response and cell cycle, was upregulated 22-fold. Expression of TOS4 is induced in G1 by bound SBF (Swi4-Swi6 cell cycle box binding factor) [29, 30]. The SBF factors bind the promoters of genes with roles in G1/S events, including DNA replication, bud growth, and spindle pole complex formation, as well as the general activities of mitochondrial function, transcription, and protein synthesis [31]. DUN1 has also been shown to regulate the expression of HUG1 or YML058w-a, a protein involved with the DNA damage checkpoint response, controlling replication arrest; it was upregulated 16-fold and 73-fold at 10 μg/ml and 100 μg/ml, respectively [32].

Similarly, DUN1 has been shown to regulate double-strand break repair, resulting in activation of DNA damage repair genes, which is a major mechanism for providing genomic stability. Critical DNA repair genes were

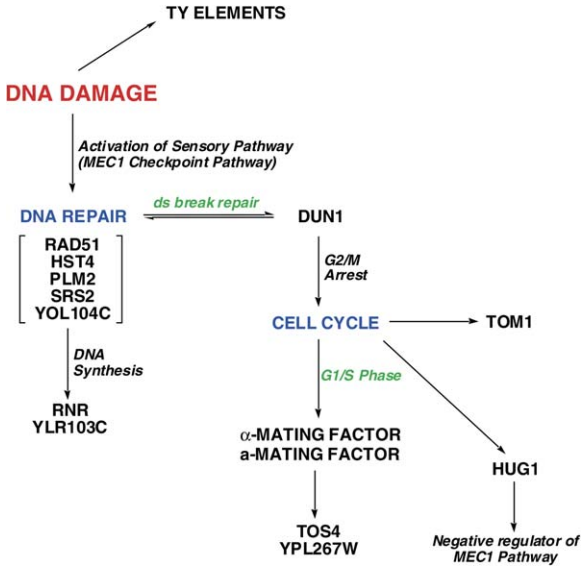

Figure 4. Correlation of Gene Changes from a Transcriptional Array Profile Showing General Activation of the MEC1 Checkpoint Pathway

upregulated in response to azinomycin B treatment. The DNA strand exchange protein RAD51, which is involved in recombinational repair of double-strand breaks [33], was enhanced in expression by 3-fold. Deletion of this gene has been demonstrated to give rise to the accumulation of double-strand breaks, and the resulting lesions are both radiation sensitive and defective in gene conversions [34]. PLM2, a transcription factor involved with plasmid stability and maintenance, was upregulated in its expression, exhibiting a dose-dependent response [29]: mRNA expression of this gene product was enhanced almost 3-fold and 9-fold at 10 μg/ml and 100 μg/ml, respectively. HST4 (YDR191W), a member of the SIR2 family of protein deacetylases [29], was increased in expression by 9-fold. This protein has a variety of biological roles, including double-strand break repair, silencing of telomeres, cell cycle progression, radiation resistance, genomic stability, and involvement in short-chain fatty acid metabolism [35]. Likewise, expression of SRS2 (YJL092W), a DNA helicase and DNA-dependent ATPase involved in DNA/double-strand break repair by nonhomologous end-joining [36, 37], was upregulated more than 3-fold, and YBL009W, a protein with strong similarity to the DNA damage response element, was enhanced 4-fold.

Genes involved in DNA synthesis and replication were also affected by azinomycin exposure, consistent with the DNA damage and crosslinking effects demonstrated for the agent in vitro. For example, the expression of the chromosomal DNA replication protein YLR103C [38, 39] was upregulated more than 6-fold. The expression of ribonucleotide reductase subunits RNR1 and RNR3 was upregulated 3-fold. These subunits comprise components of the ribonucleotide-diphosphate reductase (RNR) large subunit, which catalyzes the rate-limiting step in dNTP and DNA synthesis, and which is tightly regulated by DNA replication and DNA damage checkpoint pathways [40]. DUN1 null mutants, for example, have been shown to not only be defective in DNA

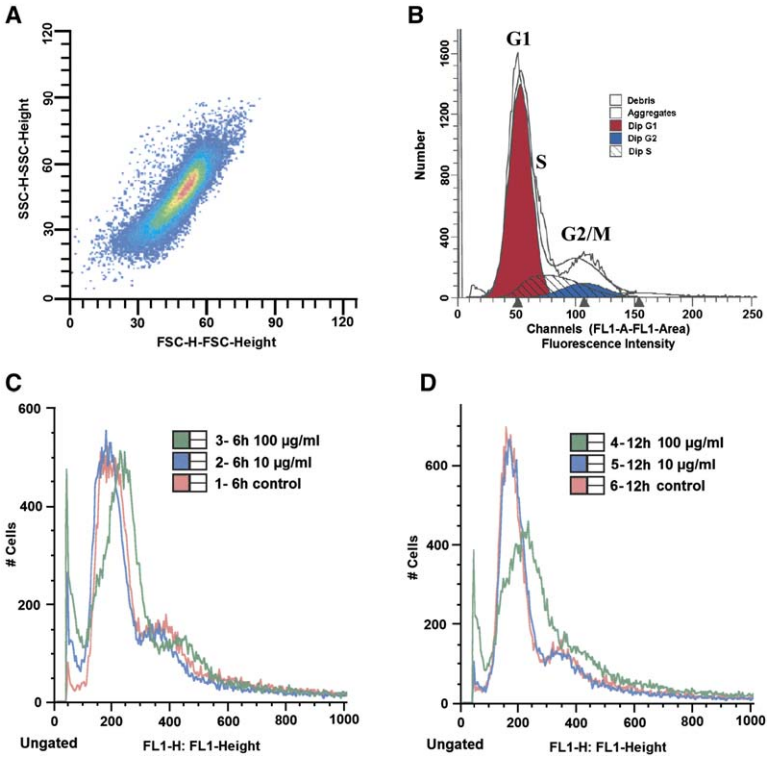

Figure 5. Cell Cycle Effects Observed with Azinomycin B Treatment

(A) Wild-type yeast cells stained with Sytox Green.

(B) Cell cycle analysis of wild-type yeast cells.

(C) Cell cycle profile of yeast cultured for 6 hr, control (pink); 10 µg/ml azinomycin (blue); and 100 µg/ml azinomycin (green).

(D) Cell cycle profile of yeast cultured for 12 hr, control (pink); 10 µg/ml azinomycin (blue); and 100 µg/ml azinomycin (green).

damage repair, but also to be defective in DNA damage-responsive induction of RNR genes (*Saccharomyces* Genome Database [SGD]).

Cell Cycle Effects

When the gene-profiling results are integrated, the transcriptional effects of azinomycin B treatment in yeast suggest a G1/S phase shift. In order to corroborate these findings, we examined the effects of azinomycin B on the yeast cell cycle. Yeast cells were treated with azinomycin B at concentrations of 10 µg/ml and 100 µg/ml for 6 hr and 12 hr, respectively, and were examined by flow cytometry (Figure 5) [41]. Figure 5A shows wild-type yeast cells stained with Sytox Green. Figure 5B depicts the cell cycle analysis of these cells. Figures 4D and 5C show the cell cycle profile of yeast cells at 6 hr and 12 hr, with and without azinomycin B treatment. When compared to the wild-type cells, azinomycin B-treated cells exhibited a predominant S phase shift. A decrease in the G2/M population was apparent at 12 hr at both concentrations of agent.

Confirmation of Selected Gene Changes by RT-PCR

Validation of the transcriptional effects induced by azinomycin B treatment was demonstrated by semi-quantitative reverse transcriptase (RT)-PCR of an arbitrary subset of the gene changes, including HUG1, RAD51, TOM1, and PLM2. The procedure, while less sensitive and quantitative than real-time PCR, is capable of confirming a transcriptional response that is marked by the presence or absence of a transcriptional effect. Normalization of the RNA/cDNA was based on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that was unaffected by drug exposure. Total RNA was extracted from control cells and cells treated with azinomycin B (10 µg/ml and 100 µg/ml). First-strand cDNA synthesis of the DNA was performed, followed by PCR analysis. The exponential phase of each PCR product was estimated by varying the number of PCR cycles for each template or gene of interest [42]. PCR products were stained with SYBR Green, and fluorescence was measured with a microplate reader (Figure 6).

Significance

Azinomycins A and B are natural products that possess promising antitumor activity. Central to the biological action of these molecules is the presence of two electrophilic carbons present within the epoxide moiety and the structurally and functionally unique aziridino[1,2-*a*]pyrrolidine core substructure. Evaluation of the azinomycins in vitro has demonstrated that the molecules bind to DNA within the major groove and form covalent interstrand crosslinks. The exquisite ability of these natural products to functionalize

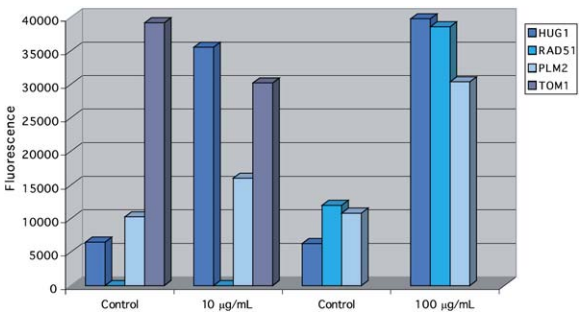

Figure 6. Semiquantitative Reverse Transcriptase-PCR Analysis of the Genes HUG1, RAD51, PLM2, and TOM1

and append to DNA notwithstanding, the cellular target of these molecules in vivo has yet to be established. In efforts to address this issue, we have examined the cellular localization pattern of azinomycin B in *Saccharomyces cerevisiae*, which paralleled that of the DNA intercalator propidium iodide.

Additionally, genomic DNA isolated from drug-treated cells showed significant shearing of the DNA, and experiments with oligonucleotide microarrays revealed transcriptional effects that were closely associated with DNA damage and repair. Genes involved in DNA synthesis and the cell cycle were altered in their expression, reflecting an S phase shift. This effect was further substantiated in flow cytometry experiments. Some genes differentially expressed in response to azinomycin B treatment were also induced by calicheamicin γ_1I or methyl methanesulfonate exposure, although a significant number of the genes that were effected by azinomycin B were not common to these other agents. Transcriptional profiling could prove to be a definitive tool in the study of the mechanism of action of DNA-damaging agents.

These results provide the first demonstration of the in vivo actions of azinomycin B. While the experiments presented here are consistent with the proposed role of the drug as a DNA crosslinking agent, definitive proof for this mechanism must come from experiments that can conclusively demonstrate drug•DNA adduct formation.

Experimental Procedures

Instrumentation and General Methods

Yeast cell cycle experiments were performed with a Becton-Dickinson FACS Calibur flow cytometer. RT-PCR reactions were carried out with an MJ Research PT600 PCR machine, and fluorescence measurements were made with a Biotek FL800 fluorescence microplate reader. Cellular localization studies were conducted with a Zeiss microscope, and the Affymetrix platform was used in all GeneChip experiments. Unless otherwise specified, biochemical reagents were obtained from Sigma Biochemicals (St. Louis, MO).

Organisms

The yeast *S. cerevisiae* wild-type strain (#404; BY4741; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) was obtained from Dr. Michael Kladde at the Department of Biochemistry, Texas A&M University. *Streptomyces sahachiroi* (NRRL 2485) was obtained from the American Type Culture Collection (ATCC; Manassas, VA).

Culture Conditions

Saccharomyces cerevisiae was maintained on YPD plates and subsequently cultured at 30ºC and 250 rpm in YPD medium [43]. *Streptomyces sahachiroi* was initially cultured on GYM plates until sporulation, typically 5–7 days at 28ºC. GYM agar plates contained the following components per liter: glucose monohydrate, 4 g; yeast extract, 4 g; malt extract, 10 g; $CaCO_3$, 2 g; agar, 12 g; and tap water; adjusted to pH 6.8 with 1 M NaOH prior to sterilization [44]. A starting culture of *Streptomyces sahachiroi* in PS5 medium (100 ml) was prepared by inoculation of a loop of spores from the GYM plates. PS5 medium was prepared from 5 g/l Pharmamedia (yellow cotton seed flour, Traders Protein; Memphis, TN) and 5 g/l soluble starch, adjusted to pH 7.0. After 24 hr of growth at 30ºC and 250 rpm, 25 ml of the starting culture was used to inoculate 500 ml PS5 in 2 liter baffled Erlenmeyer flasks. The cultures were grown for 64 hr at 30ºC and 250 rpm [44].

Azinomycin B Isolation

The natural product was isolated with slight modification of literature protocols [4, 45]. After fermentation, culture broths of *Streptomyces sahachiroi* were harvested by centrifugation (8000 rpm). The supernatant was extracted once with an equal volume of chloroform. The chloroform extract was concentrated to 5 ml and diluted with hexane (30 ml). The resulting suspension was centrifuged (3000 rpm), giving a white azinomycin-containing precipitate that was further purified by washing with ether (10 ml), followed by dissolution in chloroform (50 ml). Hexane was added gradually, and with the initial addition of hexane, a precipitate was generated and discarded. Further addition of hexane resulted in the precipitation of azinomycin B as a white, amorphous solid. The azinomycin was collected by centrifugation and stored under anhydrous diethyl ether at −80ºC.

Nuclear Localization Experiments

Yeast cells were grown at 30ºC in liquid culture to an OD_{600} of 1.0. Isolates (1 ml) were centrifuged, and the cell pellets were resuspended in 1 ml sterile Dulbecco's phosphate-buffered saline. The cells were centrifuged and treated with (1) 100 μg/ml and 4000 μg/ml azinomycin (live cells), (2) 500 μg/ml naphthoate [5, 46], and (3) 1 μg/ml propidium iodide. Propidium iodide-stained cells were fixed by the addition of 70% ethanol (final volume, 30 min of incubation at room temperature) prior to resuspension in Dulbecco's phosphate-buffered saline and treatment with propidium iodide. After 4 hr of incubation at room temperature, the cells were centrifuged, the supernatant was removed, and the cells were resuspended in Dulbecco's phosphate-buffered saline. The cells were viewed on slides with a Zeiss fluorescence microscope, and the images were captured digitally at 1000× magnification. The conditions for image capture were held constant.

Genomic Analysis

Saccharomyces cerevisiae was cultured in 10 ml aliquots at 30ºC to an OD_{600} of 1.0 and was subsequently treated with either 100 μl azinomycin B or ethanol (to give final concentrations of 10 μg/ml and 100 μg/ml, respectively). The cells were cultured for an additional 12 hr with shaking and were centrifuged, and the genomic DNA was isolated as follows. The cells were resuspended in 500 μl solution consisting of 1 M sorbitol and 0.2 M EDTA (pH 7.5), at which point 5 μl zymolyase (0.5 mg/ml) was added, and the suspension was incubated at 37ºC for 60 min. The cells were centrifuged, resuspended in 500 μl 50 mM Tris (pH 7.4), 20 mM EDTA, and 25 μl 20% SDS was added. After incubation of the mixture at 50ºC for 30 min, 5 μl proteinase K (10 mg/ml) and 200 μl 5 M potassium acetate were added to the solution. The reaction was placed on ice for 60 min, and the cellular debris was removed by centrifugation. The supernatant was transferred to a new tube, and one volume of isopropanol was added. The mixture was allowed to stand at room temperature for 5 min. The DNA was isolated by centrifugation (5 min) and was dissolved in 100 μl TE buffer containing 1 μl RNase. The suspension was incubated at 37ºC for 30 min, at which time the DNA was precipitated by the addition of 400 μl 5 M NH_4OAc and 200 μl isopropanol, centrifuged, washed with 1 ml 80% EtOH, dried, and dissolved in 100 μl TE buffer. The DNA, 5 μl of each sample, was analyzed by agarose gel electrophoresis and stained with SYBR Green by following the manufacturer's protocol (Molecular Probes, Invitrogen; Carlsbad, CA).

Yeast Sample Preparation and GeneChip Evaluation

Yeast cells were cultured overnight in YPD broth [43] to an OD_{600} of 1.0. Total RNA was extracted with an SDS/hot phenol extraction method. Yeast cell samples were centrifuged, and the supernatant was decanted. Each cell sample was washed with 30 ml deionized distilled water and subsequently centrifuged (repeated three times). Yeast pellets were frozen in liquid nitrogen and lysed by using the following protocol. Each cell pellet was treated with 10× high-salt solution (3 M NaCl, 200 mM Tris [pH 8.0], 100 mM EDTA) and deionized distilled water to a final volume of 700 μl. Each sample was brought to 1% SDS by the addition of 10% SDS (70 μl), at which time hot phenol (65ºC, 600 μl) was added. Samples were vortexed, incubated at 65ºC for 4 min, and chilled on ice for 2–4 min. Samples were microcentrifuged for 2 min, and the supernatant was transferred to new tubes. The phenol extraction procedure was repeated, and it was followed by extraction with a 25:24:1 phenol:chloroform:isoamyl alcohol. After centrifugation, the supernatant of each sample was transferred to new tubes. The RNA was precipitated by

the addition of ethanol to the top of each microfuge tube. Samples were mixed by vortexing, stored at 20ºC for 1 hr, and pelleted by centrifugation. The supernatant was decanted, and the pellet from each sample was washed twice with 70% ethanol. Residual ethanol was removed, and the cell pellet was resuspended in 100 μl water. The resulting total RNA was then treated with DNase: total RNA (30 μl) was incubated at room temperature with 5 μl of first-strand buffer (GIBCO-BRL cDNA Superscript Choice Kit) and 1 μl DNase I (RNase-free, Ambion). Samples were heat inactivated at 75ºC for 15 min and purified with an RNeasy kit (Qiagen). Samples (15 μg of total RNA per sample) were provided to the Texas A&M GeneChip facility, where they were amplified, biotinylated, and hybridized to GeneChips according to the protocol detailed by Affymetrix (Santa Clara, CA). Microarray data are available through the NCBI Geo Database (accession number: GSE4311).

GeneChip Evaluation by RT-PCR

RT-PCR analysis of a subset of the genes, including HUG1, RAD51, TOM1, and PLM2, was carried out as follows. Total RNA (10 μg) was extracted and transcribed into single-stranded cDNA by using the Superscript Choice system (GIBCO-BRL). The cDNA was purified with a Qiagen nucleotide removal kit. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as an endogenous amplification standard (i.e., used to normalize the amount of RNA in control and treatment samples). PCR conditions were optimized so that amplification of both GAPDH and the cDNA of interest was in the exponential phase. PCR cycles consisted of step 1: 5 min denaturation at 95ºC; step 2: 1 min of denaturation at 95ºC; step 3: 1 min of primer annealing at 55ºC; step 4: 1 min of extension at 72ºC. Steps 2–4 were repeated for the requisite number of cycles.

Primers

The following primers were used:

HUG1 (YML058W-A): forward: 5′-ATGACCATGGACCAAGGCCT TAACC-3′, reverse: 5′-TTAGTTGGAAGTATTCTTACCAATG-3′
RAD51 (YER095W): forward: 5′-CGGATGTGAAAAAACTAAGG GAGAG-3′, reverse: 5′-TCTTAACTGATGATCGGCGTTATAG-3′
TOM1 (YDR457W): forward: 5′-AACAGCTCGGTTCCATGAATTT GAT-3′, reverse: 5′-TAATTACGGAACGTGCTAGCATTCC-3′
PLM2 (YDR501W): forward: 5′-TTGGCTAAAGGTGAAACTGTT ACTT-3′, reverse: 5′-GCGAAAGATTCTTCTTCATTAATGC-3′
GAPDH (YJR009C): forward: 5′-ACATTGACATCGCCATTGACT CCAC-3′, reverse: 5′-TTTCATCGTAGGTGGTTTCCTTGTT-3′

After PCR, each reaction was visualized by agarose gel electrophoresis (15 μl per lane) and stained with ethidium bromide. The resulting PCR products were sliced out of the gel and dialyzed against TAE buffer [47]. The dialyzed gel pieces were stained with SYBR Green (Molecular Probes, Invitrogen) and placed in a 96-well plate, and fluorescence was measured with a Biotek FL800 microplate reader (filters: 485/30 for excitation and 528/20 for emission).

Cell Cycle Analysis by Flow Cytometry

Yeast cells were grown at 30ºC in liquid culture to an OD_{600} of 1.0 and were exposed to azinomycin B (10 and 100 μg/ml, in triplicate) for 6 and 12 hr. The yeast population was asynchronous. Cells were centrifuged, the medium was removed, and the cells were washed with 10 mM Tris (pH 7.5). The cells were fixed by treatment with 70% ethanol for 1 hr at room temperature. Cells were centrifuged, the supernatant was discarded, and the pellet was incubated with 0.5 ml 50 mM Tris (pH 7.5) supplemented with 2 mg/ml RNase A at 37ºC for 2–12 hr. After incubation, cells were centrifuged, and the supernatant discarded. The cells were resuspended in 0.2 ml proteinase solution (5 mg/ml pepsin, 4.5 μl/ml concentrated HCl in H_2O) for 15–20 min at 37ºC. The cells were centrifuged, the supernatant was discarded, and the cells were resuspended in 0.5 ml Tris (pH 7.5) and 1× Sytox Green (100 nM; Molecular Probes, Invitrogen), followed directly by the analysis step [48].

Supplemental Data

Supplemental Data including quantitative distribution analyses of yeast cell cycle populations are available at http://www.chembiol.com/cgi/content/full/13/5/485/DC1/.

Acknowledgments

C.M.H.W. would like to acknowledge the Welch Foundation, the Elsa Pardee Foundation, Texas A&M University, and the National Institutes of Health Chemistry Biology Interface Training Grant (T32 GM008523) for support of this work. R.S.C. would like to acknowledge the National Cancer Institute (R01 CA65875) for funding this project. The authors would like to thank Dr. Richard Long at the Department of Oceanography for use of a Zeiss microscope, Mr. Rader Jensen for preliminary work with the yeast azinomycin profile, and Dr. Michael Kladde at the Department of Biochemistry for helpful discussions.

Received: March 31, 2005
Revised: February 17, 2006
Accepted: February 21, 2006
Published: May 29, 2006

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