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DNA Protein Cross-Links Produced by NSC 652287, a Novel Thiophene Derivative Active Against Human Renal Cancer Cells

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ABSTRACT

2, 5-bis(5-Hydroxymethyl-2-thienyl)furan (NSC 652287), is a representative of a series of thiophene derivatives that exhibit potent and selective antitumor activity against several tumor cell lines in the National Cancer Institute Anticancer Drug Screen. NSC 652287 has noticeable activity for the renal cell lines and produces cures in certain corresponding xenografts. The cellular mechanisms of action of NSC 652287 were therefore investigated in this study in greater detail. The most sensitive renal carcinoma cell line, A498, exhibited cell cycle arrest in G_0-G_1 and G_2-M at 10 nM NSC 652287, with increased p53 and $p21^{WAF1}$ protein. At higher concentrations, NSC 652287 still induced p53 elevation but with $p21^{WAF1}$ reduction and massive apoptosis. These results collectively suggested that NSC 652287 induced DNA damage. Using alkaline elution techniques, we found that NSC 652287 induced both DNA-protein and DNA-DNA cross-links with no detectable DNA single-strand breaks. These DNA-protein cross-links (DPC) persisted for at least 12 h after drug removal and their frequency was correlated with cytotoxicity in the renal cell lines studied. The most sensitive cells (A498) produced the highest DPC followed by the cell line with intermediate sensitivity (TK-10). DPC were minimal in the two resistant cell lines, ACHN and UO-31. Nonetheless, a similar degree of DPC occurred at doses imparting equitoxic effects. These results indicate that DNA is a primary target for the novel and potent anticancer thiophene derivative, NSC 652287. NSC 652287 did not cross-link purified DNA or mammalian topoisomerase I suggesting the importance of active metabolite(s) for the cross-linking activity.

The tricyclic bis-thiophene, 2, 5-bis(5-hydroxymethyl-2-thienyl)furan (NSC 652287) (Fig. 1), is a representative of a series of thiophene derivatives that demonstrate potent activity against a subset of cell lines in the National Cancer Institute (NCI) Anticancer Drug Screen. NSC 652287 has selective activity against many cell lines within the lung, colon, ovarian, and renal subpanels of the NCI Anticancer Drug Screen with GI_{50s} (50% growth inhibition) between 10 and 400 nM. The most sensitive cell line to thiophene NSC 652287 is the A498 renal cell carcinoma, and curative antitumor activity can be observed against this cell line grown as a nude mice xenograft model (Carter et al., 1996; Rivera et al., 1999). This observation is of particular interest because there are only few therapeutic options with reliable activity against metastatic renal cell carcinomas. The pattern of differential growth inhibition and cytotoxicity when examined using the COMPARE algorithm (Paull et al., 1989) was different from that evoked by any of 176 standard anticancer agents. Because the COMPARE algorithm can potentially identify a compound's mechanism of action or molecular target (Paull et al., 1992, 1995; Monks et al., 1997), the unique cytotoxicity pattern of the thiophene derivatives suggests a unique mechanism of action.

We recently found a direct correlation between drug sensitivity and the ability of several renal carcinoma cell lines to generate radioactivity covalently bound to cellular contents after exposure to [^{14}C]NSC 652287 (Rivera et al., 1999). These results suggested that the capacity of sensitive cell lines to transform NSC 652287 to a species capable of interacting with cellular macromolecules (e.g., protein or DNA) be the basis for the selective antitumor activity of this class of thiophene derivatives.

We examined the effects of the bis-thiophene, NSC 652287 on cellular DNA in relation to cell cycle progression, induc-

[1] Both authors contributed equally to this work.

ABBREVIATIONS: NSC 652287, 2, 5-bis(5-hydroxymethyl-2-thienyl)furan; DAPI, 4,6-diamidino-2-phenylindole; SRB, sulforhodamine B; PI, propidium iodide; PAGE, polyacrylamide gel electrophoresis; DPC, DNA-protein cross-links; ISC, interstrand cross-links; CPT, camptothecin; GI_{50}; 50% growth inhibition; top1, topoisomerase I.

tion of apoptosis, and induction of p53 and $p21^{WAF1}$ protein levels. We demonstrate here that the induction of DNA and protein cross-linking by NSC 652287 are related to this drug's selective cytotoxicity and cell cycle arrest in the renal cell lines studied. We therefore identify the chemotype exemplified by NSC 652287 as capable of generating DNA-interactive species that may be considered a lead for useful agents in renal carcinomas.

Materials and Methods

Drugs and Reagents

Cell culture reagents were purchased from Quality Biological, Inc. (Gaithersburg, MD) except for fetal bovine serum, which was purchased from Hyclone Lab., Inc. (Logan, UT). The bis-thiophene, NSC 652287 (purity >99%; as determined by liquid chromatography-mass spectrometry) was obtained from the repository of the National Cancer Institute-Frederick Cancer Research and Development Center (NCI-FCRDC). Dr. Michael Cava, University of Alabama, originally submitted NSC 652287 to the NCI. Stock solutions (10 mM) were prepared in dimethyl sulfoxide and frozen at −70°C. Immediately before drug incubations, NSC 652287 was diluted in cell culture medium to the desired drug concentration. [^{14}C]-thymidine (53.6 mCi/mmol), and methyl-[^{3}H]thymidine (80.9 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Tetrapropylammonium hydroxide (40% aqueous solution) was obtained from RSA Corporation (Danbury, CT). 4,6-Diamidino-2-phenylindole (DAPI) and all other reagents were obtained from Sigma (St. Louis, MO).

Cell Lines and Culture

The human renal tumor cell lines were obtained from the NCI Repository at NCI-FCRDC, Frederick, MD. The identities, sources, derivation, and morphological and immunocytochemical characteristics of the cell lines have been previously published (Alley et al., 1988; Monks et al., 1991; Stinson et al., 1992). Cultures were in RPMI medium (Quality Biological Inc.) supplemented with 10% fetal bovine serum (Hyclone Lab.), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Quality Biological Inc.). Cells were grown at 37°C in an atmosphere of 5% CO_2/95% humidified air.

Cytotoxicity Determination

Cytotoxicity data were obtained from the NCI Anticancer Drug Screen. The sulforhodamine B (SRB) assay measures total protein content of the surviving cells. Determinations were made after 2 days of continuous drug exposure. SRB assay results were confirmed by 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino)-carbonyl]-2H-tetrazolium hydroxide (XTT) assay as described by Scudiero et al. (1988). Cells were plated in 96-well flat-bottomed plates at a density of 1500 cells/well and incubated for 24 h at 37°C in a humidified 5% CO_2/95% air atmosphere. Serial concentrations of thiophene in dimethyl sulfoxide were added to the wells and sensitivity determined 48 h after addition of the compound. Dye reduction was measured in a spectrophotometer with dual wavelength mode (450 nm and 650 nm reference). Phenazine methosulfate was used to facilitate XTT formazan formation. The percentage of control was calculated and the GI_{50} determined.

Cell Cycle Analysis

A498 cells were grown in T165 cm^2 tissue culture flasks to 60 to 70% confluence. Cells were incubated with fresh medium containing NSC 652287 for up to 72 h. At the end of each incubation period, 1 to 2×10^6 cells were fixed in 70% cold ethanol and stored at −20°C until analysis. Fixed cells were treated with phosphate-citrate buffer [0.2 M Na_2HPO_4 and 0.1 M citric acid (pH 7.8)] for 15 min at room temperature to extract low mw DNA (Darzynkiewicz et al., 1994). Cells were incubated with 100 U/ml RNase and 50 μg/ml propidium iodide (PI; 10% Nonidet P-40 in PBS) for 30 min at room temperature. Stained nuclei were then analyzed for DNA-PI fluorescence using a Becton Dickinson FACScan (San Jose, CA). An excitation source of 488 nm was achieved using a 15-mW air-cooled argon-ion laser. Fluorescence emission was collected through a 585/42 band pass filter for PI. Fifteen thousand events were collected for each sample. CELLQuest software, version 3.1 (Becton Dickinson), was used for acquisition of data. ModFit *LT* software, version 1.01 (Verity Software House, Inc., Topsham, ME) was used for data analysis.

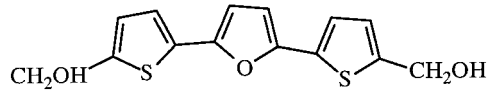

Fig. 1. Chemical structure of the thiophene NSC 652287 [2, 5-bis(5-hydroxymethyl-2-thienyl)furan].

Morphological Assessment of Cell Death

Exponentially growing cells were treated with 10 or 100 nM NSC 652287 for 24 h. Cells were harvested, washed with PBS, and fixed in 1% paraformaldehyde for 30 min on ice. Cells were kept resuspended in 70% ethanol until staining. After washing with PBS, the cells were incubated with DAPI (2 μg/ml) at 37°C for 30 min. Cells were visualized using an inverted drop technique in a fluorescent microscope.

TABLE 1

Cytotoxicity of NSC 652287 in renal carcinoma cell lines from NCI Anticancer Drug Screen

Cytotoxicity was assessed by the SRB assay at 48 h of drug exposure. Data were obtained from six to seven independent experiments. A full set of data including data for the other cell lines of the NCI Anticancer Screen are available at http://dtp.nci.nih.gov/.

Cell Line[a]	GI_{50}	TGI[b]	LC_{50}[c]
		μM	
A498 (Wt)	0.017 ± 0.003	0.035 ± 0.010	0.144 ± 0.04
TK-10 (m)	0.032 ± 0.008	0.232 ± 0.116	>100
ACHN (Wt)	22.76 ± 13.08	94.52 ± 5.47	>100
UO-31 (Wt)	41.71 ± 7.61	88.58 ± 11.41	>100

[a] p53 status indicated in parenthesis (Wt: wild-type p53; m: mutant p53) (O'Connor et al., 1997).
[b] TGI, drug concentration required for total growth inhibition.
[c] LC_{50}, drug concentration required for 50% cell killing.

TABLE 2

Cell cycle alterations and apoptotic DNA fragmentations induced by NSC 652287 in human A-498 renal carcinoma cells

Values correspond to percentage of cells in each population (G_0-G_1, S, G_2-M, or SUB-G_1) as determined from flow cytometry data. ModFit *LT* software, version 1.01 (Verity Software House, Inc., Topsham, ME) was used for data analysis.

	Hours			
	6[a]	24	48	72
Control				
G_0-G_1	46.3 ± 2.51	48.9 ± 0.87	66.5 ± 1.11	75.7 ± 1.36
S	45.6 ± 1.41	44.9 ± 0.68	20.8 ± 0.96	17.1 ± 1.42
G_2-M	6.20 ± 1.00	4.86 ± 0.55	12.0 ± 1.10	6.21 ± 0.55
SUB-G_1	1.53 ± 1.10	1.32 ± 0.39	0.70 ± 0.17	0.97 ± 0.38
10 nM				
G_0-G_1	41.8 ± 0.97	35.5 ± 1.86	36.6 ± 1.64	38.2 ± 0.59
S	51.6 ± 0.45	18.2 ± 1.14	16.8 ± 0.35	14.3 ± 0.91
G_2-M	5.02 ± 0.50	44.7 ± 1.91	45.1 ± 2.45	43.6 ± 1.97
SUB-G_1	1.70 ± 0.62	1.67 ± 0.55	1.60 ± 0.61	3.43 ± 1.75
100 nM				
G_0-G_1	45.8 ± 1.65	36.8 ± 0.47	25.7 ± 2.26	16.2 ± 0.31
S	48.4 ± 1.01	30.9 ± 0.88	26.7 ± 1.40	18.1 ± 1.62
G_2-M	3.80 ± 0.21	22.9 ± 0.45	20.7 ± 1.62	14.9 ± 0.68
SUB-G_1	1.30 ± 0.50	9.30 ± 0.36	26.9 ± 5.35	51.0 ± 2.25

[a] A498 cells in exponential growth were treated with 10 or 100 nM NSC 652287 and harvested at the indicated time points.

Immunoblotting

A498 cells were grown in 100 mm^2 tissue culture dishes to 60 to 70% confluence. Cells were incubated with fresh medium containing NSC 652287. At selected time intervals (6, 24, and 48 h), cells were lysed in 250 μl of buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM EGTA, 20 mM NaF, 50 mM B-glycerophosphate, 10% glycerol, 1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM PMSF, and 1 mM sodium orthovanadate]. Lysates were centrifuged at 12,000 rpm for 10 min, and supernatants were stored at −70°C until analysis. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (1970) using 10 or 14% Tris-glycine gels for p53 and p21, respectively. Protein concentration was determined by the Coomasie blue method (Pierce Chemical Co., Rockford, IL) using BSA as a standard. Twenty micrograms of protein were loaded/well. Transfer to Immobilon-*P* (polyvinylidene difluoride) membranes (Millipore, Bedford, MA) was effected in a tank system (Hoefer Scientific Instruments, San Francisco, CA) for 1.25 h at 1 Amp, using 3-cyclohexylamino-1-propanesulfonic acid (pH 11.0, 10% MeOH), as the transfer buffer. Membranes were blocked in Tris buffered saline-Tween (0.1%) containing 5% nonfat dried milk for at least 1 h and then probed with the primary antibody for 1 h, and for 1 h with secondary antibody (1:25,000 dilution) at 20°C. Proteins were immunodetected using standard enhanced chemiluminescence techniques (Amersham Life Science, Arlington Heights, IL). A monoclonal mouse anti-p53 (Ab-6; Calbiochem, La Jolla, CA) and polyclonal rabbit anti-p21 (Ab-6; Calbiochem) antibody were used at a 1:1000 and 1:200 dilution, respectively. Equal protein loading was assessed by blotting with anti-β-actin antibody.

Determination of DNA Damage by Alkaline Elution

Alkaline elution was performed to assess DNA damage by detecting DNA-protein and DNA-DNA cross-links, and DNA strand breaks as previously described (Kohn et al., 1981; Bertrand and Pommier, 1995; Kohn, 1996). Before alkaline elution and drug treatments, cells were radiolabeled with 0.02 μCi/ml of [^{14}C]thymidine for 1 to 2 doubling times at 37°C and then chased in nonradioactive medium overnight. After drug treatments, cells were scraped in Hanks' balanced salt solution, counted, and aliquots of cell suspensions were placed in drug-containing ice-cold Hanks' balanced salt solution. After alkaline elution, filters were incubated at 65°C with 1N HCl for 45 min and then 0.04 M NaCl were added for an additional 45 min. Radioactivity in all fractions was measured with a liquid scintillation analyzer (Packard Instruments, Meriden, CT).

DNA-Protein Cross-Links (DPC). DPC were analyzed under nondeproteinizing, DNA-denaturing conditions using protein-adsorbing filters (polyvinylchloride-acrylic copolymer filters, 0.8 μm pore size; Gelman Science, Ann Harbor, MI), and LS10 lysis solution (2 M NaCl, 0.2% Sarkosyl, and 0.04 M disodium EDTA, pH 10). All cell suspensions were irradiated with 30 Gy. The DNA was eluted from filters with tetrapropylammonium hydroxide-EDTA, pH 12.1, without SDS at a flow rate of ≈0.035 ml/min. Fractions were collected at 3-h intervals for 15 h. DPC frequencies were calculated according to the bound to one terminus model formula (Kohn et al., 1981).

Determination of DNA Strand Breaks. DNA single-strand breaks were assessed by alkaline elution under deproteinizing, DNA denaturing conditions. Briefly, after treatment, radiolabeled cells were harvested at 4°C, loaded onto polycarbonate filters (2 μm pore size; Poretics, Livermore, CA) and lysed with SDS buffer (0.1 M glycine, 0.025 M EDTA, 2% w/v SDS, and 0.5 mg/ml proteinase K, pH 10). The lysis solution was washed from filters with 0.02 M EDTA, pH 10, and the DNA was eluted with tetrapropylammonium hydroxide-EDTA, pH 12.1, containing 0.1% SDS at a flow rate of 0.035 ml/min into five fractions at 3-h intervals.

Determination of DNA Interstrand Cross-Links (ISC). The induction of ISC was assessed by alkaline elution under deproteinizing and DNA denaturing conditions. Immediately after drug treat-

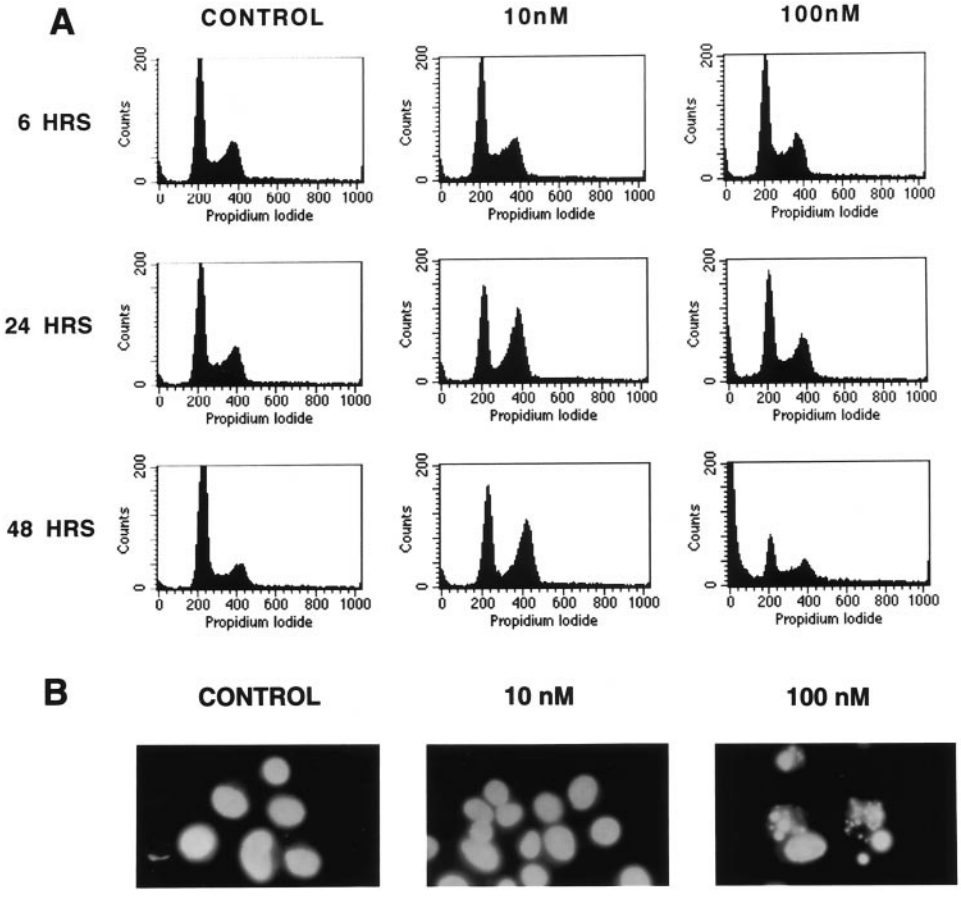

Fig. 2. A, cell cycle arrest and apoptosis induced by NSC 652287 in A498 renal carcinoma cells. A498 cells in exponential growth were treated with 10 or 100 nM NSC 652287 and harvested at the indicated time points. Cell cycle profiles were obtained with a Becton-Dickinson FACScan. B, morphological assessment of apoptosis induced by NSC 652287 in A498 cells. Cells were treated with the indicated doses for 24 h, harvested, and fixed in 1% paraformaldehyde. Cells were kept suspended in ethanol until staining. After washing with PBS, cell suspensions were incubated with DAPI (2 μg/ml) at 37°C for 30 min and examined by fluorescence microscopy.

ment, cells were harvested, irradiated with 3 Gy, and loaded onto polycarbonate (nonprotein adsorbing) filters. Cells were lyzed with SDS buffer (0.1 M glycine, 0.025 M EDTA, 2% w/v SDS, and 0.5 mg/ml proteinase K, pH 10). DNA was eluted with tetrapropylammonium hydroxide-EDTA, pH 12.1, containing 0.1% SDS at a flow rate of 0.035 ml/min into five fractions at 3-h intervals.

DNA Relaxation Assays

Native supercoiled simian virus 40 (SV40) DNA was incubated with various concentrations of NSC 652287 in the presence of topoisomerase I (top1; Life Technologies-BRL, Gaithersburg, MD) for 1 h at 37°C (Pommier et al., 1987). Reaction mixtures were separated in1% agarose gels in 1× TBE buffer (89 mm Tris · borate and 2 mm EDTA, pH 8.3) and stained with ethidium bromide for visualization under UV light. Camptothecin (CPT) was used as a positive control.

Top I-Mediated DNA Cleavage Using Oligonucleotide Cleavage Assays

Cleavage assays were performed as previously described (Pommier et al., 1995). Briefly, a duplex oligonucleotide (36 mer) was 3′-end labeled with α-[^{32}P]cordycepin and reacted with DNA top1 (Life Technologies-BRL) in the presence or absence of drug. After 15 min, reactions were stopped by adding SDS (0.5% final concentration). Maxam-Gilbert loading buffer (98% formamide, 0.01 M EDTA, 1 μg/ml xylene cyanol, and 1 μg/ml bromophenol blue) was then added to samples, which were electrophoresed in 16% polyacrylamide gel containing 7 M urea in TBE buffer. Imaging was performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Results

NSC 652287 Induces G_2-M Cell Cycle Arrest at Low Doses and Apoptosis at Higher Doses. The cellular effects of the bis-thiophene derivative, NSC 652287 (Fig. 1) were studied first in A498 renal carcinoma cells that are among the most sensitive cell lines in the NCI Anticancer Drug Screen to this agent (see Table 1; for complete data see: http://dtp.nci.nih.gov/). Figure 2 demonstrates that incubation with 10 nM NSC 652287 caused cell cycle arrest with accumulation of cells at the G_2-M phase after 24 h. Thereafter, cells accumulated both in G_0-G_1 and G_2-M phases. This cell cycle arrest was sustained for at least 72 h. Higher concentrations of NSC 652287 (100 nM) produced an increase in propidium iodide fluorescence in the sub-G_0-G_1 area of the histogram, which suggested the induction of DNA fragmentation and apoptosis (Table 2). These FACS analysis results are consistent with growth inhibition experiments, and with a decrease in cell number and viability at 100 nM NSC 652287 as determined by trypan blue exclusion (data not shown).

Figure 2B shows an assessment of NSC 652287-induced cell death by fluorescent microscopy after staining with DAPI. Apoptotic nuclear condensation and nuclear fragmentation was observed by 24 h of continuous treatment with 100 nM thiophene. These observations suggest that apoptosis is the mechanism leading to cell death in A498 cells treated with 100 nM NSC 652287.

NSC 652287 Increases p53 and Induces Dose-Dependent Effects on p21^{WAF1} Protein Levels. The induction of cell cycle arrest at both G_0-G_1 and G_2-M phases by NSC 652287 prompted us to examine p53 and p21^{WAF1} protein levels in drug-treated A498 cells. The A498 renal carcinoma cell line is known to have wild-type p53. Figure 3 demonstrates that p53 was elevated after exposure to 10 and 100

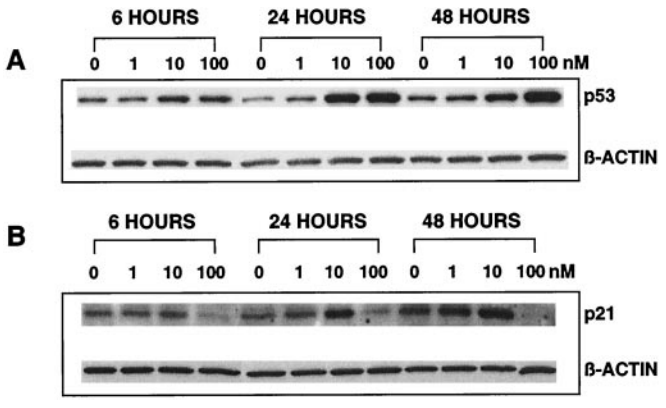

Fig. 3. Elevation of p53 and p21^{WAF1} protein levels in A498 renal carcinoma cells treated with NSC 652287. Exponentially growing cells were treated with the indicated NSC 652287 concentrations and harvested at 6, 24, and 48 h of continuous drug exposure. Cells were lyzed and 20 μg of protein were separated by SDS-PAGE in 10% (for p53) or 14% (for p21^{WAF1}) gels, and transferred to Immobilon-*P* membranes. Mouse monoclonal anti-p53 and rabbit polyclonal anti-p21 antibodies were used. β-actin was used as a loading control.

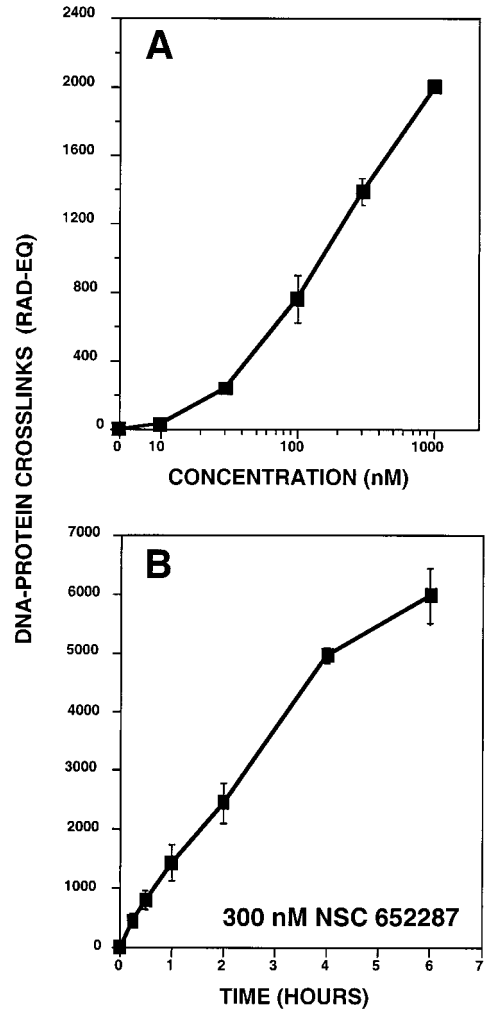

Fig. 4. Production of DPC in A498 renal carcinoma cells treated with NSC 652287. DPC were measured by alkaline elution. A, concentration-dependence: DPC were measured after 1 h of drug treatment. B, time-dependence for DPC formation in A498 cells treated with 300 nM NSC 652287. Bars represent standard errors for at least three independent experiments.

nM NSC 652287. p53 protein levels were elevated as early as 6 h after exposure to drug. Thus, the elevation of p53 was coincident with the observed cell cycle arrest at 10 nM NSC 652287 and with apoptosis at 100 nM drug concentration.

$p21^{WAF1}$ protein levels were then examined because p53-dependent cell cycle arrest is mediated by $p21^{WAF1}$. Figure 3B demonstrates that $p21^{WAF1}$ protein levels increased after 24 h exposure of A498 cells to 10 nM NSC 652287. These results are consistent with a role of $p21^{WAF1}$ in the p53-dependent cell cycle arrest produced by exposure to low concentrations of NSC 652287.

By contrast, $p21^{WAF1}$ protein levels decreased within 6 h after exposure to 100 nM NSC 652287 (Fig. 3B). Even after 24 and 48 h of drug exposure, $p21^{WAF1}$ protein levels were less than in untreated cells in spite of elevated p53 protein levels in the cells treated with NSC 652287 (Fig. 3). Thus, in A498 cells, apoptosis induced by 100 nM NSC 652287 was associated with p53 elevation and $p21^{WAF1}$ reduction.

NSC 652287 Induces Both DNA-Protein and DNA-DNA Cross-Links in Cells. The cell cycle arrest and elevation of p53 and $p21^{WAF1}$ proteins observed in the A498 cells treated with NSC 652287 suggested a DNA damage response. Assessment of DNA damage was done by alkaline elution, which allows the detection and quantitation of DPC, DNA strand breaks, and DNA ICSs (reviewed in Kohn, 1996). Figure 4 shows that NSC 652287 produced DPC in a concentration- and time-dependent manner. DPC were detectable after 1 h exposure to concentrations as low as 30 nM (Fig. 4A). The induction of DPC followed an almost linear progression as a function of time with high DPC frequency after 6 h of drug treatment.

Drug-induced DPC are usually associated either with DNA-DNA cross-links in the case of alkylating agents or with DNA strand breaks in the case of topoisomerase inhibitors (Kohn, 1996). Alkaline elution experiments were then performed to investigate whether other DNA lesions were associated with the DPC induced by NSC 652287. Figure 5 demonstrates that NSC 652287 also induced DNA-DNA cross-links. Such DNA-DNA cross-links retard the elution of DNA that had been irradiated with 3 Gy immediately after drug treatment (Kohn, 1996). DNA-DNA cross-links were detectable after 1 h of drug exposure at concentrations as low as 10 nM. Elution experiments performed in the absence of irradiation indicated that NSC 652287 did not induce detectable DNA strand breaks at concentrations that produced high frequencies of both DNA-protein and DNA-DNA cross-links (Fig. 5).

We then studied the reversal kinetics of the DNA cross-links. Figure 6 shows that DPC were readily detectable 15 min after drug addition and were not reversible within 12 h after drug removal. Thus, NSC 652287-induced DNA cross-links are persistent after drug removal.

DPC Induction Correlates with Sensitivity of Renal Carcinoma Cell Lines to NSC 652287. The formation of DPC was assessed in the four renal carcinoma cell lines from the NCI Anticancer Drug Screen to determine the relationship between DPC formation and cytotoxicity. As shown in Table 1, A498 cells are the most sensitive to NSC 652287. ACHN and UO-31 cells are resistant to the compound and TK-10 cells exhibit intermediate sensitivity. Figure 7 shows the formation of DPC for the four cell lines. DPC were not detectable in the two resistant cell lines (ACHN and UO-31) after 1 h treatment with NSC 652287, whereas DPC formation was high in A498 cells and low in TK-10 cells under similar conditions (Fig. 7A).

Formation of DPC was then assessed after different incubation times (Fig. 7B). DPC formation was minimal in the resistant ACHN and UO-31 cells even after 18 and 48 h of drug exposure, respectively. The incubation times were selected as the time needed for 50% of the parent compound to disappear from the culture medium (Rivera et al., 1999). The formation of DPC was also investigated at concentrations close to the GI_{50} values. Each of the four cell lines was incubated for 48 h with NSC 652287 close to the GI_{50} concentration, as determined by the SRB assay (Table 1). Figure 7C demonstrates that, under these conditions, the formation of DPC was comparable in the four cell lines. These experiments indicate a relationship between NSC 652287-induced DPC frequency and cytotoxic activity of NSC 652287.

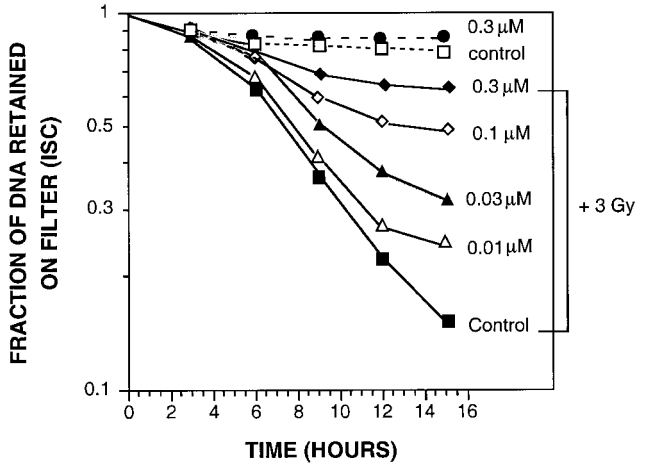

Fig. 5. Production of DNA ISC by NSC 652287. A498 renal carcinoma cells were treated with the indicated NSC 652287 concentrations for 1 h. For the ISC assays, cells were scraped in iced Hanks' balanced salt solution immediately after 1 h of drug treatment and irradiated on ice with 3 Gy before alkaline elution. Drug concentrations for the ISC assays were: 0.3 μM (♦), 0.1 μM (◇), 0.03 μM (▲), 0.01 μM (△), or 0 (no drug treatment; ■). Lack of production of DNA strand breaks by NSC 652287 was evaluated in cells treated with NSC 652287 in the absence of irradiation. Drug concentrations for the DNA strand break assays were: 0.3 μM (●) or 0 (no drug treatment; □).

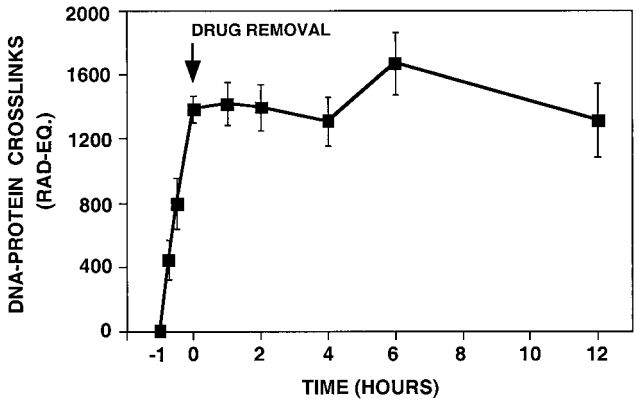

Fig. 6. Persistence of the DPC induced by NSC 652287 (300 nM) in A498 cells. DPC formation was measured after 15-, 30-, and 60-min drug treatments. DPC reversal was measured at the indicated times after removing NSC 652287 by three washings of the cells by centrifugation/resuspension in drug-free medium. Bars represent standard errors for at least three independent experiments.

NSC 652287 Does Not Affect Top1-Mediated DNA Relaxation or Cleavage Activity. Because our data indicate that NSC 652287 is a potent inducer of DNA cross-links in renal cancer cells, we wished to determine whether a direct interaction of NSC 652287 could be determined with purified DNA. In an effort to elucidate the nature of the formation of DPC, we investigated possible interactions of NSC 652287 with DNA in the absence and presence of eukaryotic top1. Figure 8 shows that NSC 652287 does not affect top1-mediated relaxation of supercoiled SV40 DNA (Fig. 8A). This suggests that NSC 652287 is not a DNA intercalator. In the absence of top1, NSC 652287 did not affect the migration of SV40 DNA or of a short oligonucleotide indicating no detectable DNA-DNA cross-linking under these conditions. Figure 8B demonstrates that NSC 652287 does not affect top1 activity, as measured by cleavage of an oligonucleotide containing a unique top1 cleavage site (Pommier et al., 1995) in the absence or presence of the selective top1 inhibitor CPT. Thus, NSC 652287 exhibited no detectable DNA or protein interaction in these assays.

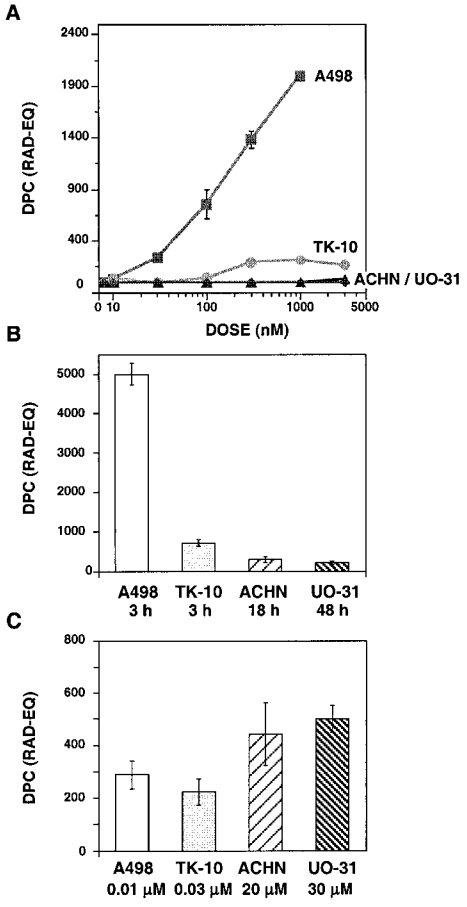

Fig. 7. Relationship between NSC 652287-induced DPC and cytotoxicity in the renal carcinoma cell lines from the NCI Anticancer Drug Screen. A498 cells are the most sensitive cells to thiophene NSC 652287. TK-10 cells demonstrate intermediate sensitivity, whereas ACHN and UO-31 cells are resistant (see Table 1). A, DPC were measured by alkaline elution after 1 h of treatment with NSC 652287. B, comparison of DPC formation at different times after treatment with 300 nM NSC 652287 in the four cell lines. Incubation times (in hours) are indicated for each cell line, and were determined as the time required for 50% disappearance of the parental compound from the cells' culture medium (Rivera et al., 1999). C, DPC formation at concentrations of NSC 652287 close the GI_{50} values for the four cell lines (see also Table 1). Cells were treated to for 48 h. Bars indicate standard error for at least three independent determinations.

Discussion

NSC 652287 is a representative of a novel class of thiophene derivatives discovered in the NCI Anticancer Drug Screen for their activity against the subpanel of cell lines derived from renal cancer, a disease for which present chemotherapy has limited activity. The present study shows that thiophene NSC 652287 is a potent inducer of apoptosis at submicromolar concentrations. Apoptosis was associated with p53 elevation and decrease of $p21^{WAF1}$ protein levels in A498 cells. By contrast, lower NSC 652287 concentrations induced elevation of both p53 and $p21^{WAF1}$ and cell cycle arrest (Fig. 3 and Table 2). A decrease of $p21^{WAF1}$ in spite of p53 elevation at NSC 652287 concentrations that induced apoptosis was probably due to $p21^{WAF1}$ degradation during apoptosis. $p21^{WAF1}$ is indeed a substrate for caspases (Gervais et al., 1998).

Although A498 cells have wild-type p53 and are very sensitive to NSC 652287, two of the most resistant kidney cell lines of the NCI Anticancer Drug Screen, ACHN and UO-31, also have wild-type p53 (O'Connor et al., 1997; Table 1). Thus, sensitivity to NSC 652287 appears independent of p53 in the cell lines examined.

p53 elevation and G_2-M arrest prompted us to evaluate whether thiophene NSC 652287 induced DNA damage. The present study shows that NSC 652287 induces both DPC and DNA-DNA cross-links but no DNA strand breaks. These cross-links persisted for at least 12 h after drug removal and

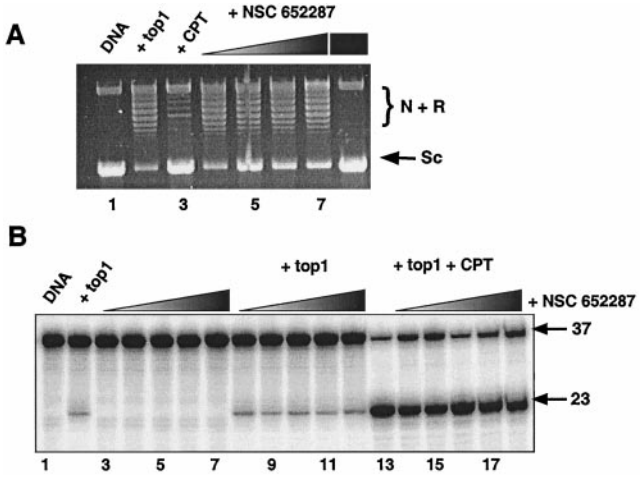

Fig. 8. NSC 652287 does not exhibit detectable interaction with purified DNA and/or calf thymus top1. A, effect of NSC 652287 on SV40 plasmid relaxation. Native supercoiled SV40 DNA was incubated with NSC 652287 (0.1 μM in lane 4, 0.3 μM in lane 5, 1 μM in lane 6, and 3 μM in lanes 7 and 8) in the presence of top1 for 1 h at 37°C temperature. No top1 was present in lanes 1 and 8. Reaction mixtures were separated in 1% agarose (TBE) and stained with ethidium bromide. CPT was used as a positive control. N, R & Sc: nicked, relaxed, and supercoiled DNA, respectively. B, lack of effect of NSC 652287 on duplex oligonucleotide DNA in the absence (lanes 3–7) or presence of top1 (lanes 8–12 and 14–18). An oligonucleotide (36 mer) containing a unique strong top1 cleavage site was 3′-end-labeled with α-[^{32}P]cordycepin. Reactions were carried out in the presence or absence of top1 at room temperature for 1 h. NSC 652287 concentrations were 0.01, 0.1, 1, 10, and 100 μM in lanes 3–7, 8–12, and 13–17. Reaction mixtures were separated by denaturing PAGE in 16% gels and visualized by PhosphorImager analysis. CPT (1 μM) was used as a positive control and to stimulate top1-mediated DNA cleavage.

their frequency was correlated with NSC 652287 cytotoxicity. These results indicate that one of the primary targets of thiophene NSC 652287 is DNA and that this drug acts as a DNA cross-linking agent.

Thiophene NSC 652287 had no effect on purified DNA, which suggests that an active drug metabolite might be responsible for reacting with DNA and/or cellular proteins. Sausville and coworkers (Rivera et al., 1999) found a correlation between NSC 652287 metabolism and cytotoxicity in the renal cancer cell lines of the NCI Anticancer Drug Screen used in the present study. The structure of NSC 652287 (Fig. 1) suggests that the methanol groups could be readily converted into reactive aldehydes that could form covalent bonds with protein and/or DNA nucleophilic groups. Thus, thiophene NSC 652287 probably represents a prodrug for a novel class of bifunctional DNA- and/or protein-alkylating agent.

Thiophene NSC 652287 was selected for further characterization in part because of its behavior in the NCI's Drug Screening Program (Rivera et al., 1999). As described elsewhere (Monks et al., 1991), this screen use a panel of 60 different human tumor cell lines to assess cytostatic effect after a 48-h drug exposure. The patterns of cytotoxicity that emerge can be analyzed by various pattern recognition algorithms, including COMPARE (Paull et al., 1995). This analysis estimates a correlation coefficient of a test substance's pattern of differential cytotoxicity with those of previously tested compounds. Antiproliferative agents with a common target or mechanism of action have been found in a number of cases to have similar patterns, including, for example, tubulin-directed agents (Paull et al., 1992; Solary et al., 1993), topoisomerase inhibitors (Leteurtre et al., 1994; Kohlhagen et al., 1998), and antimetabolites (Jayaram et al., 1992). Thus, there is considerable interest in novel patterns of cytotoxicity, as is manifested by thiophene NSC 652287.

Novel patterns of compound action in the NCI Anticancer Drug Screen appear to arise in two distinct ways. First, agents may combine with high affinity for a molecular target distinct from those recognized by standard antineoplastic agents. Examples would include flavopiridol, now known as a potent inhibitor of cyclin-dependent kinases (Sedlacek et al., 1996), UCN-01, another protein kinase antagonist (Seynaeve et al., 1994), and japlakinolide or cucurbitacin (Bubb et al., 1994; Duncan et al., 1996), which target the actin cytoskeleton. Alternatively, selective uptake or differential capacity for or metabolism of compounds has been demonstrated in the case of ellipticiniums (Acton et al., 1994; Vistica et al., 1996) or benzothiazoles. The thiophene NSC 652287 would appear to represent a notable example of the latter case, where differential capacity to metabolize the compound results, as demonstrated here, in the capacity to create DNA damage.

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