In vitro evaluation of dimethane sulfonate analogues with potential alkylating activity and selective renal cell carcinoma cytotoxicity

Susan D. Mertins,1,2 Timothy G. Myers,2 Susan L. Holbeck,2 Wilma Medina-Perez,1 Elaine Wang,1 Glenda Kohlhagen,3 Yves Pommier,2 and Susan E. Bates1

1Cancer Therapeutics Branch, 2Developmental Therapeutics Program, and 3Laboratory of Molecular Pharmacology, National Cancer Institute, NIH, Bethesda, Maryland

Abstract
We identified five structurally related dimethane sulfonates with putative selective cytotoxicity in renal cancer cell lines. These compounds have a hydrophobic moiety linked to a predicted alkylating group. A COMPARE analysis with the National Cancer Institute Anticancer Drug Screen standard agent database found significant correlations between the IC50 of the test compounds and the IC50 of alkylating agents (e.g., r = 0.68, P < 0.00001 for chlorambucil). In this report, we examined whether these compounds had activities similar to those of conventional alkylating agents. In cytotoxicity studies, chlorambucil-resistant Walker rat carcinoma cells were 4- to 11-fold cross-resistant to the test compounds compared with 14-fold resistant to chlorambucil. To determine effects on cell cycle progression, renal cell carcinoma (RCC) line 109 was labeled with bromodeoxyuridine prior to drug treatment. Complete cell cycle arrest occurred in cells treated with an IC50 dose of NSC 268965. p53 protein levels increased as much as 5.7-fold in RCC line 109 and as much as 20.4-fold in breast cancer line MCF-7 following an 18-hour drug exposure. Finally, DNA-protein cross-links were found following a 6-hour pretreatment with all compounds. Thus, the dimethane sulfonate analogues have properties expected of some alkylating agents but, unlike conventional alkylating agents, appear to possess activity against RCC. [Mol Cancer Ther 2004;3(7):849–60]

Introduction
Renal cell carcinoma (RCC) continues to be a therapeutic challenge because systemic treatment is effective in only a fraction of patients with advanced stage disease (1). Previously, we identified 16 compounds in the National Cancer Institute (NCI) Anticancer Drug Screen that have particular sensitivity in the renal cell line subpanel (2). A subset of these, a family of five dimethane sulfonates (DMS), were chosen for further study because they were cytotoxic to slowly growing renal cells in vitro, a potentially useful property for agents targeted to indolent RCC. In this report, we present the initial studies characterizing their activities.

The DMS analogues have a superficial structural similarity to the sulfonates busulphan, hepsulfam, and treosulfan. This resemblance suggests that the mechanism of action of the new compounds may relate to alkylating activity. Hepsulfam and busulphan readily form DNA-protein cross-links (DPCs; ref. 3), while treosulfan alkylates DNA, although presumably by the formation of epoxides, a mechanism distinct from the other sulfonates (4). Yoshi 864, the core DMS moiety common to all five renal selective agents, was evaluated in Phase II clinical trials against several different malignancies (5, 6). It has been shown to alkylate a pyrimidine in vitro (7).

The alkylating agents used clinically are a diverse group, targeting different sites within the DNA molecule and, in some cases, attacking other biologically relevant molecules (8). For example, carmustine [1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)] alkylates at the N7 position of guanine, while nitrogen mustards such as chlorambucil and melphalan target the N7 position. In contrast, a BCNU derivative has been shown to alkylate tubulin (9). Notably, estramustine, an agent originally designed to be a more effective alkylating agent, had little or no such activity, but rather disrupted the microtubular architecture (10). Thus, distinct and sometimes overlapping sites of action characterize the alkylating agents.

We sought to evaluate the DMS compounds in several assays in which alkylating agents would have predictable effects. For example, it might be expected that the DMS analogues would be cross-resistant to cell lines selected with known alkylating agents. Furthermore, known alkylating agents cause G2 and G1 arrest (11), and it would be of interest to determine if the DMS analogues cause similar cell cycle alterations. In particular, it has been shown that melphalan can cause delays in transition from G2-M to G0-G1 at sublethal concentrations (12) and that both ethyl methane sulfonate (13) and Yoshi 864 (14), at lethal doses, cause a complete arrest of cycling cells. These findings suggest that there are multiple targets for alkylating agents, which are disrupted at distinct cell cycle checkpoints.
We chose to evaluate p53 induction as a universal property of alkylating agents that is presumably mediated by DNA damage due to alkylation, although this has not been rigorously studied. Cell-specific differences in p53 induction have been noted (15-17). Recent evidence suggests that the cellular repair capabilities may mediate the induction or stability of p53. It was first thought that DNA strand breaks alone (15) were sufficient to induce p53, but now it has been shown that human tumor cells proficient for mismatch repair induce p53 in response to O\textsubscript{6}-methylguanine lesions following N\textsubscript{2}-methyl-N\textsuperscript{'}-nitro-N-nitrosoguanidine treatment, while deficient cells do not stabilize p53 under the same conditions (18).

As a final strategy, we asked whether the DMS compounds caused DPC formation that occurs following exposure to most alkylating agents. Nitrogen mustards, nitrosoureas, and sulfonates have been shown to cause this type of damage (19). However, DPC is not a type of damage unique to alkylating agents because doxorubicin can also cause their formation (20).

Thus, we undertook a series of studies that evaluate a novel family of DMS analogues that have significant activity in renal cell lines and structurally resemble the alkylating agent, busulfan. These studies were not designed to ask what the mechanism of RCC selectivity might be, but rather to evaluate similarity to known activities of alkylating agents.
agents. We analyzed NCI Anticancer Drug Screen cytotoxicity fingerprints by COMPARE analysis (21), evaluated their cytotoxicity in the NCI Yeast Anticancer Drug Screen in yeast strains mutant for cell cycle control and DNA repair genes, evaluated cross-resistance patterns in cell lines resistant to chlorambucil, evaluated cell cycle progression following exposure to the DMS analogues, determined whether p53 was elevated, and measured DPC formation.

Materials and Methods

Cell Lines

Two renal cell lines derived from primary RCC biopsies were used in these studies: 109 (p53 wild-type), derived from a papillary RCC; and 161, derived from clear-cell RCC (22-25). The breast cell line MCF-7 was obtained from the NCI Anticancer Drug Screen. These cell lines were maintained in improved MEM or RPMI (Biofluids, Gaithersburg, MD). All media were supplemented with 10% FCS, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 units/mL streptomycin (Biofluids and Life Technologies, Inc., Gaithersburg, MD).

The Walker 256 rat mammary carcinoma cell lines sensitive and resistant to chlorambucil were a gift of Dr. Kenneth Tew (Philadelphia, PA) and were grown in supplemented DMEM (Biofluids, Gaithersburg, MD). The resistant cell lines (~20-fold resistant to chlorambucil; ref. 26) were cultured without cytotoxic compound at least 1 week prior to assay.

Cytotoxic Agents

All DMS compounds were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, NCI, NIH (Bethesda, MD), solubilized in DMSO (Sigma Chemical Co., St. Louis, MO), and stored at −20°C. Their structures are shown in Fig. 1A. Cis-diaminedichloroplatinum (II), BCNU (or carmustine), and busulfan were obtained from Sigma Chemical. Melphalan (NSC 8806), chlorambucil (NSC 3088), and Yoshi 864 (NSC 102627) were obtained from the NCI Anticancer Drug Screen. Doxorubicin hydrochloride was obtained from Pharmacia, Inc. (Kalamazoo, MI). The structures of the alkylating agents used in the comparison studies are shown in Fig. 1B.

Cytotoxicity Assays

Methodology for measurement of cytotoxicity has been reported previously (27). Adherent cell lines (RCC lines and cis-diaminedichloroplatinum (II) resistant and parental lines) were seeded into 96-well plates (Costar/Corning Inc., Corning, NY) at a concentration of 2 × 10⁴ cells per well 1 day prior to drug addition. Each compound concentration was evaluated in quadruplicate. In brief, cells were incubated 96 hours with drug, fixed with 10% (w/v) trichloroacetic acid (Mallinckrodt, Paris, KY), and stained with sulforhodamine B (Sigma Chemical). Sulforhodamine B was solubilized with 10 mmol/L unbuffered Tris (Molecular Biologicals, Inc., Columbia, MD) prior to measurement of the A₅₆₄ in a microplate spectrophotometer (Bio-Rad, Hercules, CA).

Nonadherent cell lines (chlorambucil-resistant and parental lines) were treated similarly, except that cell growth was measured by formation of formazan product from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical). After 96-hour drug exposure, the cells were incubated with 20 μg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide diluted in DMEM (4 hours, 37°C, 5% CO₂). The cells were sedimented by centrifugation, and the supernatant was carefully removed. DMSO was added to solubilize the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide product. The A₅₆₄ was measured using a Bio-Rad microplate spectrophotometer.

Percentage growth inhibition was calculated for both assays using the following formula: A₅₆₄ (control cells) − A₅₆₄ (treated cells) / A₅₆₄ (control cells) × 100. For each compound, the results were graphed using a semilogarithmic plot, and the IC₅₀ was extrapolated.

Cell Cycle Analysis Using Bromodeoxyuridine Labeling

RCC line 109 was plated (3 × 10⁵ cells per 100 mm Petri dish) to obtain logarithmic growth prior to cell cycle analysis following drug exposure using a previously described methodology (28). In brief, bromodeoxyuridine (BrdUrd; Sigma Chemical) was added directly to the culture medium (10 μmol/L) and incubated for 30 minutes at 37°C. Baseline BrdUrd labeling was evaluated for 30 minutes. The remaining cells were washed and exposed to an IC₅₀ or lethal dose of the test compound or diluent. At selected time points, the cells were trypsinized (Life Technologies), washed in PBS (without magnesium and calcium; Biofluids, Rockville, MD) containing 1% bovine serum albumin (ICN Biomedicals, Inc., Aurora, OH), and fixed in cold 70% ethanol (Warner-Graham Co., Cockeysville, MD). The DNA was denatured with 2 N HCl (Mallinckrodt) with 0.5% Triton X-100 (v/v, Sigma Chemical), and the cells were neutralized, washed, and resuspended in 0.1 mol/L sodium tetraborate (pH 8.5; Fisher Scientific, Fair Lawn, NJ). The fixed and denatured cells were washed, resuspended in 0.5% Tween 20 (Sigma Chemical) and 1.0% bovine serum albumin (w/v) in PBS, and counted. The cells were incubated with FITC-labeled anti-BrdUrd (Becton Dickinson, San Jose, CA) for 30 minutes at room temperature, washed, and resuspended in 5 μg/mL propidium iodide (Sigma Chemical). Bivariate measurement of fluorescent anti-BrdUrd and total DNA content was performed using a FACSort flow cytometer equipped with an argon laser (488 nm; Becton Dickinson).

Mathematical modeling supplied by the Modfit software package (Verity Software House, Inc., Topsham, ME) was used to determine the distribution of control cells throughout the cell cycle compartments. These gates were applied to treated cells.

Immunoblotting

The RCC line 109 and MCF-7 cell lines were plated for logarithmic growth 1 day prior to 18-hour drug exposure (concentrations of test compounds and doxorubicin ranged...
from one ninth to nine times the IC$_{50}$. Subsequently, the cells were washed with cold PBS and harvested with lysis buffer [10 mmol/L Tris (Molecular Biologicals), 1.0 mmol/L EDTA (Quality Biologicals, Gaithersburg, MD), 50 mmol/L NaCl (Quality Biologicals), 0.5% deoxycholate (Sigma Chemical), 0.5% NP40 (Sigma Chemical), 0.5% SDS (Sigma Chemical), 1.0 mmol/L phenylmethylsulfonyl fluoride (Bio-Rad), 0.15 mg/mL aprotinin (ICN Biomedicals), and 0.15 mg/mL leupeptin (ICN Biomedicals)]. The whole cell lysate was heated to 100°C for 10 minutes and centrifuged (1,000 × g, 30 minutes, 4°C). Protein concentrations were measured with Bio-Rad Protein Assay Reagent. Samples were electrophoresed on SDS-PAGE gels (National Diagnostics, Atlanta, GA) and transferred to nitrocellulose (Millipore Corp., Bedford, MA; ref. 29). Standard immunoblotting techniques were used for the p53 antibody [1:1,000 dilution (AB-6); Oncogen, Cambridge, MA; ref. 30]. Loading controls [using anti-tubulin antibody, 1:1,000 dilution (DM1A); Sigma Chemical] were evaluated and were consistent with measured protein levels and Ponceau staining (data not shown). Visualization of specific bands was accomplished by probing with an immunoperoxidase-labeled secondary immunoglobulin antibody directed to the specific class and developing the membrane with the enhanced chemiluminescence reagent (DuPont, Wilmington, DE). Densitometry was performed using the IP Lab Gel (Scanalytics, Inc., Fairfax, VA) and Adobe Photoshop (Adobe, San Jose, CA) software packages installed on a Macintosh Power PC integrated with an Epson scanner (Epson America, Long Beach, CA).

**Measurement of DPCs**

Nondeproteinizing, DNA-denaturing alkaline elution was used to determine the amount of DPC as described previously (31). Renal (RCC line 161) and leukemia (K562 and MOLT4) cells were labeled with 0.02 μCi/mL $^{14}$C-thymidine for one to two doubling times at 37°C. All cells were chased in nonradioactive medium at least 18 hours before drug treatment. After a 6-hour drug treatment (1 and 10 μmol/L), an aliquot of cells (~25,000 dpm) from each treatment flask was transferred to tubes containing cold HBSS and placed on ice. The samples were irradiated on ice with 30 Gy prior to elution. Cells were gently layered onto polyvinylchloride-acrylic copolymer filters (Metricel DM-800, Gelman Sciences, Ann Arbor, MI) and lysed using a solution containing 0.2% sodium sarkosyl-2 mol/L NaCl-0.04 mol/L EDTA (pH 10.0) in the absence of proteinase K. After one wash with 0.02 mol/L EDTA (pH 10), the DNA was eluted with tetrapropylammonium hydroxide-EDTA (pH 12.2) without SDS. Fractons were collected at 3-hour intervals for 15 hours. DPC determinations were made by comparing the radioactivity in the eluent with that of a standard containing known quantities of DPC (data not shown). Because the DMS analogues (Fig. 1A) had binding to the cell cycle studies using the Minitab statistical software (Minitab, State College, PA). Pearson correlation coefficients were also determined with this software package.

**NCI Yeast Anticancer Drug Screen**

The NCI Yeast Anticancer Drug Screen was performed at the Fred Hutchinson Cancer Research Center (Seattle, WA; refs. 33, 34). Experimental design and publicly available data (including the results for the DMS compounds reported here) are available at http://dtp.nci.nih.gov/yacds/default.html.

**Results**

**COMPARE Analysis of DMS Analogaes and Standard Anticancer Agent Database of the NCI Anticancer Drug Screen**

We demonstrated previously that the renal active patterns of the DMS analogues were different from that of standard agents (five standard agents: doxorubicin, paclitaxel, 5-fluorouracil, cisplatin, and etoposide; ref. 2). As expected, there were no statistically significant Pearson correlation coefficients in any of the pairwise comparisons (data not shown). Because the DMS analogues (Fig. 1A) had a structural similarity to the alkylating agent busulfan (Fig. 1B), we were interested in determining whether this potential mechanism of action could be confirmed by a COMPARE analysis. Previous studies have shown that the cytotoxicity activity patterns of the 60 cell line panel of the NCI Anticancer Drug Screen can identify compounds with similar mechanisms of action (21). We performed a series of COMPARE analyses that calculated Pearson correlation coefficients between the IC$_{50}$ values of the unknown compounds and the IC$_{50}$ values of known standard agents as a measure of similarity. The results are shown in Fig. 2 (top). There were similarities between all DMS analogues and representative alkylating agents Yoshi 864 (NSC 102627), busulfan (NSC 750), chlorambucil (NSC 3088), and melphalan (NSC 8806). There was little similarity to
These compounds are presented because of structural similarities to the DMS analogues. Only triethylenemelamine and thiotepa had higher Pearson correlation coefficients (than those presented) of the standard agents in the database. There were also similarities among the DMS compounds.

To evaluate whether there was a unique component to the DMS fingerprints that could be distinguished from the alkylating activity, we calculated the partial correlation after subtracting the component of the correlation attributable to the alkylating activity. The average alkylating agent fingerprint (12 representative alkylating agent fingerprints) was used to represent the average alkylating activity factor. The DMS analogues were clustered in Fig. 2 (top) to imply a superficial relatedness among the DMS analogues. Interestingly, NSC 281612, a DMS analogue with modest renal selectivity, is the least similar among the DMS family (as measured by Pearson correlation coefficient). We calculated the partial correlation with the remaining component and a particular alkylating agent (i.e., Yoshi 864, busulfan, chlorambucil, melphalan, and BCNU). The results are shown in Fig. 2 (bottom). As expected, similarities with the standard alkylating agents were removed. It is notable that a unique component remained among the DMS compounds, signaled by the Pearson correlation coefficient remaining at >0.5.

In sum, by COMPARE analysis, the DMS analogues have similar cytotoxicity fingerprints to known alkylating agents: the greatest similarity to nitrogen mustards and the least similarity to the nitrosurea, BCNU. In addition, the agents under study may also have activity unrelated to that of conventional alkylating agents.

**Evaluation of Cross-Resistance of DMS Analogues in Chlorambucil-Resistant Lines**

Because the structures of the DMS compounds suggested alkylating activity and the COMPARE analysis suggested that a potential mechanism of action of the DMS analogues overlapped with that of chlorambucil, we examined the relative resistance of the DMS analogues in the Walker rat carcinoma lines selected for resistance to chlorambucil. The parental and resistant lines were plated 1 day prior to drug exposure to achieve logarithmic growth. After 96 hours of drug exposure, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed to quantitate growth inhibition. IC$_{50}$ values were determined, and relative resistance of the selected lines was calculated. The results are detailed in Table 1. Similar to a previous report (26), the resistant cell lines were 14.3-fold cross-resistant to chlorambucil, 3.9-fold to busulfan, and unchanged in response to BCNU. The degree of cross-resistance for NSC 268965 and NSC 281612 was similar to that for chlorambucil (i.e., 10.0- and 11.7-fold, respectively). Although the IC$_{50}$ values for Yoshi 864, the compound containing only the DMS, is consistent with the mean IC$_{50}$ value determined in the NCI Anticancer Drug Screen, little cross-resistance was found (1.9-fold). The selected cell lines were cross-resistant to the remaining DMS analogues but similar to the lower levels found for busulfan (3.9-fold). DMS analogues with similar hydrophobic moieties, NSC 281613, NSC 281617, and NSC 281817, were 4.0- to 6.8-fold more resistant in the selected cell line. To determine whether the observed cross-resistance of these three DMS analogues could be attributed to the hydrophobic ring structure, we evaluated the effect of NSC 281610. No cross-resistance was found.

![Figure 2. Analysis of DMS compounds and known alkylating agents. Top, Pearson correlation coefficients were determined for the DMS analogues and standard alkylating agents and were clustered. DMS analogues were found to be similar to standard alkylating agents. Bottom, a mean alkylating agent fingerprint was first calculated from 12 standard agents and subtracted from the fingerprint of each DMS analogue. A unique fingerprint remained.](mct.aacrjournals.org)
In Vitro Studies of Dimethane Sulfonate Analogues

Table 1. Resistance of known alkylating agents and DMS in chlorambucil-resistant lines

<table>
<thead>
<tr>
<th>Known alkylating agents</th>
<th>Walker Sensitive IC_{50} (µmol/L)</th>
<th>Walker Resistant IC_{50} (µmol/L)</th>
<th>Relative Resistance (Fold Change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carmustine</td>
<td>28 ± 0.3</td>
<td>22 ± 0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>Chlorambucil</td>
<td>0.3 ± 0.01</td>
<td>4.3 ± 0.02</td>
<td>14.3</td>
</tr>
<tr>
<td>Busulfan</td>
<td>20 ± 0.1</td>
<td>75 ± 0.4</td>
<td>3.9</td>
</tr>
<tr>
<td>Yoshi 864 (NSC 102627)</td>
<td>62 ± 0.3</td>
<td>118 ± 0.1</td>
<td>1.9</td>
</tr>
<tr>
<td>DMS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSC 72151</td>
<td>0.14 ± 0.001</td>
<td>0.69 ± 0.005</td>
<td>4.9</td>
</tr>
<tr>
<td>NSC 268965</td>
<td>0.031 ± 0.002</td>
<td>0.31 ± 0.001</td>
<td>10.0</td>
</tr>
<tr>
<td>NSC 280074</td>
<td>0.06 ± 0.006</td>
<td>0.5 ± 0.005</td>
<td>8.3</td>
</tr>
<tr>
<td>NSC 281610*</td>
<td>18 ± 0.2</td>
<td>17 ± 0.3</td>
<td>0.9</td>
</tr>
<tr>
<td>NSC 281612</td>
<td>0.6 ± 0.007</td>
<td>7 ± 0.07</td>
<td>11.7</td>
</tr>
<tr>
<td>NSC 281613</td>
<td>0.87 ± 0.06</td>
<td>5.9 ± 0.04</td>
<td>6.8</td>
</tr>
<tr>
<td>NSC 281617</td>
<td>0.8 ± 0.003</td>
<td>5.4 ± 0.05</td>
<td>6.8</td>
</tr>
<tr>
<td>NSC 281817</td>
<td>0.8 ± 0.003</td>
<td>3.2 ± 0.04</td>
<td>4.0</td>
</tr>
</tbody>
</table>

NOTE: Cytotoxicity assays were conducted using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide as a measure of viable cells remaining after 96-hour drug exposure. Percentage survival was calculated, and an IC_{50} value was determined. Relative resistance is determined by the ratio of IC_{50} of resistant cell line to the IC_{50} of the sensitive cell line.

*Hydrophobic moiety similar to NSC 281613, NSC 281617, and NSC 281817 without the DMS group.

In sum, the DMS analogues were cross-resistant in cell lines selected with a known alkylating agent, chlorambucil. However, neither the DMS chain alone (Yoshi 864) nor the hydrophobic portion alone (NSC 281610) were cross-resistant when tested independently.

Evaluation of Cell Cycle Progression Using BrdUrd Labeling

Known alkylating agents arrest cells at the G1-S and G2-M transitions and are thought to do so because damaged DNA is detected by checkpoint proteins at this time (11). We asked whether the DMS compounds would have similar cell cycle effects. We investigated the effects of IC_{50} (a concentration at which 90% of the cells were killed in a 96-hour assay) and IC_{90} (a concentration at which 50% of the cells were killed in a 96-hour assay) concentrations of DMS analogues on a RCC line in a kinetic analysis to define any changes in cell cycle progression immediately after drug exposure. In our previous studies, we found that some DMS analogues only require a 3-hour exposure for maximum cytotoxicity (2). In the experiments reported here, we grew RCC line 109 logarithmically, labeled with BrdUrd for 30 minutes and washed thoroughly. Fresh medium containing the DMS analogue of interest or diluent was added. The progression of labeled and unlabeled cells over a 24-hour exposure time to a IC_{90} dose of NSC 268965 or NSC 281612 is shown in Fig. 3.

At the top of Fig. 3, the cell cycle histogram of the initial growing cell population is depicted. At 30 minutes, a population of BrdUrd-labeled cells was identified using gates determined by nonspecific and propidium iodide staining. The G0-G1 and G2-M populations were also separated on the same basis. The gates were fixed for all time point determinations. By 9 hours, control treated and labeled cells had progressed from S to G2-M and appeared in G0-G1. By 9 hours, unlabeled G0-G1 cells can be seen to enter S phase. In NSC 268965 treated cells, no labeled cells have progressed from S to G0-G1 at 9 hours or at any subsequent time point. Furthermore, the unlabeled G0-G1 population did not progress to S phase at any time point. For NSC 281612 treated cells, at 9- and 12-hour time points, there was no progression of labeled cells through the cell cycle. However, at 16 hours, unlabeled G0-G1 cells are shown to enter S phase. Thus, complete cell cycle arrest was found for NSC 268965 treated cells and S-phase arrest for cells treated with NSC 281612.

In contrast to Fig. 3 in which the effect of the IC_{90} was evaluated, Fig. 4A and B summarizes the effect of the IC_{50} concentration of the DMS analogues on cell cycle progression during a 24-hour time course. To quantitate the delay of the S-phase cells from G2-M to G0-G1, the percentage of control treated BrdUrd-labeled cells found in S and G2-M was calculated and reported in Fig. 4A. As expected, all control labeled cells remained in S-G2-M up to 6 hours (100%). At 8 hours and later, 80% of the labeled cells had progressed to G0-G1. This was not the case for DMS treated cells in the panel below. At 12 and 16 hours, a greater proportion of cells were delayed in S-G2-M (n = 7, P = 0.0008). Similar results were found for BCNU treated cells (IC_{50} concentration). Yoshi 864 (NSC 102627; IC_{50} concentration) had no effect on progression of labeled cells to G0-G1 (open inverted triangles). The failure to demonstrate altered cell cycle progression with Yoshi 864 contrasts with a previous report and may be due to the use of an IC_{50} concentration here rather than the higher dose examined in ref. 14.

It was also of interest to quantitate any changes in cell cycle progression from G0-G1 to S phase in these same control and drug treated cells. Thus, the percentage of unlabeled G0-G1 cells were calculated using the gates established with the BrdUrd-labeled cells and propidium...
iodide staining and were reported in Fig. 4C and D. A significant portion of control G₀-G₁ cells progressed to S phase by 12 hours as indicated by a decreased percentage of cells in G₀-G₁ (Fig. 4C). For G₀-G₁ cells treated with DMS analogues, significantly more cells remained in G₀-G₁ at 12 hours (n = 7, P = 0.008), indicating a delay at the G₁ checkpoint. However, as expected for an IC₅₀ concentration, this value declines over 24 hours, indicating that cells eventually enter S phase.

To restate, when treated with IC₉₀ concentrations of NSC 268965, growing cells completely arrest. NSC 281612 only arrested cells in S and G₂-M phases. Cells exposed to IC₅₀ concentrations of DMS analogues underwent delays in the cell cycle. The IC₅₀ concentration of BCNU also induced cell cycle arrest.

Effect of DMS Analogues on p53 Protein Levels

Because IC₉₀ concentrations of DMS analogues can cause complete cell cycle arrest in renal cells, we evaluated levels of p53, which could mediate the observed growth arrest. Logarithmically growing RCC line 109 was treated with a range of concentrations (one ninth IC₅₀, IC₅₀, and nine times IC₅₀) of five different DMS analogues or doxorubicin for 18 hours, lysed, and immunoblotted. Results for the maximum effect of the DMS compounds are shown in Fig. 5. For treated RCC line 109, induction of p53 ranged from none (NSC 281817; data not shown) to 5.7-fold (highest dose of NSC 281613). Response to doxorubicin was similar. Because this was only a small induction in p53 levels in the renal cell line, even with a compound known to induce p53, we also examined the effect of the DMS analogues on MCF-7 human breast cell line (Fig. 5). Similar changes in p53 levels were found. The fold increase ranged from none (low doses of NSC 281617; data not shown) to 37.5-fold (high dose of NSC 281613). Changes in p53 levels to doxorubicin were higher in the MCF-7 line compared with RCC line 109. To summarize, p53 levels were increased in both sensitive renal cell lines and resistant breast cell lines following DMS treatment.

Effect of DMS Analogues on DPC Formation

We examined whether the DMS analogues can induce DPC as do known alkylating agents. RCC line 161, labeled with [¹⁴C]-thymidine, was exposed to two doses of test compound (1 and 10 µmol/L) for 6 hours prior to lysis and quantitation of DPC formation. The results are shown in Fig. 6. Measurable DPCs were present at both doses; 10 µmol/L induced higher levels. NSC 281817 induced the greatest DPC, while NSC 72151 induced the fewest DPC. As a negative control, paclitaxel did not induce DPC (data not shown).

We also evaluated DPC formation in a pair of leukemia cell lines that had disparate sensitivity to the DMS analogues (Fig. 6) as a means to examine a potential mechanism of action. MOLT4 (relatively sensitive to the DMS analogues as determined by the IC₅₀ value in the NCI Anticancer...
Drug Screen) and K562 (relatively resistant) were exposed to two concentrations of DMS analogues for 6 hours prior to assessment of DPC formation. MOLT4 and K562 had equivalent DPC levels following exposure to NSC 72151, NSC 268965, and NSC 280074. Furthermore, few DPCs were present following treatment with NSC 281613 and NSC 281817 in the sensitive MOLT4 line. In contrast, greater DPC levels were found for the more resistant K562 line following exposure to these same compounds. Lastly, there was no significant positive correlation

Figure 4. Effect of DMS analogues on the cell cycle. RCC line 109 was labeled with BrdUrd prior to exposure to IC_{50} concentrations of the DMS compounds, control alkylating agent, BCNU, or diluent. Cells were harvested at subsequent time points and evaluated for cell cycle progression. Percentage labeled and unlabeled cells were identified using propidium iodide and specific FITC staining, which fixed gated populations. With time, control cells proceeded through the cell cycle and appeared in G_{0}-G_{1} (A). In contrast, cells treated with seven of eight DMS compounds were delayed in S-G_{2}-M (B) as well as cells treated with BCNU. With time, control cells proceeded through G_{0}-G_{1} to S phase (C). In contrast, cells treated with seven DMS compounds were delayed in G_{0}-G_{1} (D).

Figure 5. Effect of DMS analogues on p53 induction in a RCC line 109 and breast cell line MCF-7. Lysates were prepared following 18-hour exposure to selected DMS compounds or doxorubicin. The proteins were electrophoresed and immunoblotted with antibodies to p53. Modest induction of p53 was found in RCC line 109 following all treatments, while greater induction of p53 was found in the sensitive MOLT4 line. In contrast, greater DPC levels were found for the more resistant K562 line following exposure to these same compounds. Lastly, there was no significant positive correlation

<table>
<thead>
<tr>
<th>RCC Line 109</th>
<th>Breast Line MCF-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>No TX</td>
<td>No TX</td>
</tr>
<tr>
<td>DOX (µg/ml)</td>
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NSC 268965

4.4

NSC 281613

5.7 1.7 1.6

20.4 9.0 7.3

37.5 14.3 17.0
between IC_{50} values and DPC levels in either cell line. Thus, although we confirmed the ability of DMS compounds to induce DPC, the levels of DPC do not explain the disparate degree of resistance between MOLT4 and K562 or the renal sensitivity of the DMS analogues.

Effect of DMS Analogues in the Yeast Genetic Screen

The NCI Yeast Anticancer Drug Screen performed at the Fred Hutchinson Cancer Research Center has screened >100,000 compounds for their ability to inhibit the growth of Saccharomyces cerevisiae mutants defective in cell cycle control or DNA damage repair (analogous to defects observed in many human tumor cells; refs. 33, 34). Some of the DMS analogues (NSC 268965, NSC 280074, NSC 281612, NSC 281613, and NSC 281617), as well as the standard agents BCNU (similar to the DMS compounds by COMPARE analysis; Fig. 2) and cis-diamminedichloroplatinum (II), have been tested in the screen in concentrations ranging from 1.2 to 100 μmol/L (Fig. 7). Of note, chlorambucil was inactive in the screen, and melphalan was not tested. The alkylating agents BCNU and cisplatin [cis-diamminedichloroplatinum (II)] were highly selective, inhibiting the growth of the rad18 mutant (defective in postreplication repair) much more than any of the other yeast strains. While the DMS analogues all show the greatest inhibition in the rad18 mutants, they also inhibit the growth of the rad14 mutant (relative to the wild-type control), which is needed for nucleotide excision repair. NSC 268965, NSC 280074, and NSC 281613 inhibit the growth of the rad52 mutant, which is defective in recombinational repair of DNA damage. Because mutants in multiple DNA repair pathways are sensitive to the DMS analogues, these compounds may cause multiple types of DNA damage. In addition, NSC 268965 inhibits the growth of the mec2 (rad53) mutant, which is needed for both S-phase DNA synthesis checkpoint and G2-M DNA damage checkpoint. Because the mec2 mutant is not compromised in any of the DNA repair pathways, the sensitivity of this strain to NSC 268965 indicates either that the damage caused by this compound is poorly repaired or that NSC 268965 arrests DNA

![Figure 6](image_url)

**Figure 6.** Effect of DMS analogues on DPC formation. RCC line 161 and leukemia lines MOLT4 and K562 were exposed to DMS compounds for 6 hours following labeling with ^14^C-thymidine. Lysates were prepared and evaluated for DNA fragmentation. DPCs were induced by all DMS analogues in the RCC line 161 and the leukemia line K562. Three of five DMS analogues induced cross-links in leukemia line MOLT4.

![Figure 7](image_url)

**Figure 7.** Results from the NCI Yeast Anticancer Drug Screen. Some of the DMS compounds were tested for their ability to inhibit growth of yeast strains altered in cell cycle control and DNA repair genes. For comparison, cisplatin and BCNU are included.

synthesis. The known alkylating agents BCNU and cis-diaminedichloroplatinum (II) did not inhibit the growth of the mec2, rad14, or rad52 mutants more than the wild-type controls.

Discussion
We studied a family of five DMS compounds with renal selective activity to determine if their in vitro effects were similar to alkylating agents. A COMPARE analysis with the NCI Anticancer Drug Screen standard agent database found significant correlations between the IC50 of the test compounds and the IC50 of alkylating agents. The Seattle Yeast Project, in collaboration with the NCI Anticancer Drug Screen, evaluated the cytotoxicity of the DMS analogues on a panel of yeast mutants. Notable growth inhibition occurred in strains mutant for DNA damage repair. To determine whether these compounds had similar activities to alkylating agents, we evaluated their resistance profile in cell lines resistant to chlorambucil. Cross-resistance was observed for the DMS analogues. Cell cycle delays in S phase were noted for all but one of the DMS compounds. p53 was induced in the renal and breast cell lines. Finally, DPCs were found following pretreatment. Thus, the DMS analogues have properties that are expected of known alkylating agents in some experimental systems.

By a COMPARE analysis with standard agents of known mechanisms of action, we found high Pearson correlation coefficient between the DMS analogues and known alkylating agents such as chlorambucil and busulfan. However, we also observed using factor analysis that a portion of the DMS fingerprint is uncorrelated with alkylating agent fingerprints. This finding would be expected because the basis for selecting the DMS family for further study depended on their renal selectivity. Furthermore, a molecular target analysis (data not shown) emphasized potential for targets distinct from DNA. Future studies could be directed at these targets as an explanation for the renal selective activity.

The effects of the DMS compounds on mutant yeast strains in the NCI Yeast Anticancer Drug Screen share some features with known alkylating agents. Recently, it was shown that alkylating agents primarily inhibit the growth of postreplication DNA repair mutants, with rad18 and rad56 mutants generally showing at least 10-fold greater sensitivity than mutants in other DNA repair pathways (33). Here, we report that, in addition to the rad18 mutant, yeast strains deficient for genes involved in nucleotide excision repair (rad14) and recombinational repair (rad52) were sensitive to the DMS compounds. This indicates that these compounds have additional DNA damaging properties not shared with known alkylating agents. For NSC 268965, this damage apparently is not readily repaired because this compound also inhibits the mec2 (rad53) checkpoint deficient strain. Because the mec2 gene is needed for both the S-phase checkpoint that monitors completion of DNA synthesis and the G2-M checkpoint that detects DNA damage, the inhibition of the mec2 mutant by NSC 268965 could be due to either a block in DNA synthesis or persistent DNA damage (even with intact DNA repair systems). Notably, the mec2 mutant, which has functional DNA repair pathways, is not sensitive to known alkylating agents (which specifically inhibit rad18) or known topoisomerase poisons (which specifically inhibit rad50 and rad52 mutants).

We found that cell lines selected for resistance against chlorambucil were cross-resistant to the DMS analogues. In particular, the 10- to 11-fold resistance found for NSC 268965 and NSC 281612 were similar to levels found for chlorambucil in the Walker model. Further, the levels of cross-resistance for the remaining compounds were similar to levels for busulfan. We also found similar cross-resistance for the DMS analogues in the cisplatin-resistant lines (data not shown). However, Yoshi 864 (NSC 102627), a compound that solely contains the DMS, did not display cross-resistance in either model nor did the hydrophobic portion of the DMS analogues. This suggested that either the DMS and its presumptive alkylating activity do not play a role in the cross-resistance or it requires the phenyl ring to resemble alkylating agents in these systems. The need for a ring structure was not apparent because, in this same system, busulfan resistance can be demonstrated. It is important to note that it is not likely that the compound was inactive because the IC50 values reported here are similar to previously published ones (14). Thus, further studies are needed to confirm that the cross-resistance relates to alkylating activity.

The DMS analogues altered cell cycle progression in a manner similar to known sulfonates and in this study, BCNU. In sum, when treated with IC50 concentrations of NSC 268965, growing cells completely arrested. NSC 281612 treatment arrested cells only in S and G2-M phases. Cells exposed to IC50 concentrations of DMS analogues underwent delays in the cell cycle at the G1 and G2-M checkpoints. Thus, the findings were consistent for both sublethal and lethal concentrations and the finding from the Yeast Genetic Screen that mec2 (checkpoint) mutants are sensitive to NSC 268965. The rapid effects (by 8 hours) of the DMS analogues are consistent with our previous findings (2) that as little as 3 hours is necessary for maximum cytotoxicity. Taken together, these findings suggest that there is high affinity binding to the target(s) and it is largely irreversible. Of note, the G1 arrest, but not the G2 arrest, could be abrogated if a methyl group was added (i.e., the difference between NSC 268965 and NSC 281612 and their effects on the cell cycle). This suggests that there are at least two targets for NSC 268965.

We found that p53 protein levels were modestly increased following DMS analogue treatment. This pattern was found for sensitive renal cells and resistant MCF-7 breast cells. Previous work is conflicting or inferential regarding induction following alkylating agent exposure (15-17, 35); in fact, paclitaxel weakly induces p53 as well (36). Furthermore, we found that p21 was not induced (data
group of DMS analogues with apparent renal selectivity. These analogues may be one basis of the specificity. Block binding of nuclear factor-κB (NF-κB) sequence specificity for alkylation; for example, nitrogen mustards. An alternative explanation for the apparent renal selectivity of the DMS compounds is their ability to target the DMSO compounds to a specific site. Mechanism of action, there is little basis for the specificity resulting in intrastrand and interstrand cross-links is the mechanism of action. However, the resistant cells have increased levels of glutathione S-transferase activity. The resistant cells have an altered metabolism of these compounds. The resistant lines may account for the differences in sensitivity rather than formation alone; a kinetic study of formation and removal might reconcile these findings.

Although many of the studies presented here specifically address activities of the DMS analogues that may resemble alkylating agents, it remains possible that other targets not relating to DNA damage exist. Differences between parental and resistant lines that may underlie the observed cross-resistance have been extensively studied in the Walker rat models and may provide some insight. In particular, DNA cross-link formation to nitrogen mustards is similar in both lines as is repair of this damage (37). Additionally, one DNA repair enzyme, O6 alkyl transferase, is absent in the resistant cell line (38), thus effectively eliminating this type of damage as a possible mechanism of action. However, the resistant cells have increased levels of glutathione S-transferase activity, suggesting favorable drug detoxification. Further studies are needed to link metabolic differences to the observed cross-resistance.

One could argue that the renal selectivity of the DMS family in the NCI Anticancer Drug Screen is contradictory to the implication that these compounds are alkylating agents. No clinically useful alkylating agents have demonstrated efficacy in RCC. If alkylation damage to the DNA results in stabilization of the DNA double helix, then the resistant cell line could target the DMS compounds to a specific site. An alternative explanation for the apparent renal selective activity of the DMS analogues may reside in their sequence specificity for alkylation; for example, nitrogen mustards preferentially alkylate adjacent guanines (40). It has been shown that nitrogen mustard and melphalan can block binding of nuclear factor-κB and SPI to their canonical sites (41). Thus, hypothetically, dysfunction of transcription factors (unique to RCC) targeted to the DNA binding sites preferentially alkylated by the DMS analogues may be one basis of the specificity.

In summary, we undertook studies characterizing a group of DMS analogues with apparent renal selectivity and found that some of their properties are similar to those of known alkylating agents. Direct studies will be needed to confirm these initial findings. Interest is heightened by the in vivo efficacy (2). The compounds continue in preclinical development, with clinical trials anticipated in the future.

Acknowledgments

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References


In vitro evaluation of dimethane sulfonate analogues with potential alkylating activity and selective renal cell carcinoma cytotoxicity
