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A Novel Inhibitor Of Topoisomerase I is Selectively Toxic For A Subset of Non-Small Cell Lung Cancer Cell Lines

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Abstract

SW044248, identified through a screen for chemicals that are selectively toxic for NSCLC cell lines, was found to rapidly inhibit macromolecular synthesis in sensitive, but not in insensitive cells. SW044248 killed approximately 15% of a panel of 74 NSCLC cell lines and was non-toxic to immortalized human bronchial cell lines. The acute transcriptional response to SW044248 in sensitive HCC4017 cells correlated significantly with inhibitors of topoisomerases and SW044248 inhibited topoisomerase 1 (Top1) but not topoisomerase 2. SW044248 inhibited Top1 differently than camptothecin and camptothecin did not show the same selective toxicity as SW044248. Elimination of Top1 by siRNA partially protected cells from SW044248, although removing Top1 was itself eventually toxic. Cells resistant to SW044248 responded to the compound by upregulating CDKN1A and siRNA to CDKN1A sensitized those cells to SW044248. Thus, at least part of the differential sensitivity of NSCLC cells to SW044248 is the ability to upregulate CDKN1A.

Keywords

Non-small cell lung cancer; topoisomerase I; integrated stress response; eIF2 α ; selective toxicity

Introduction

The mutational processes that lead to cancer can also generate vulnerabilities in transformed cells that might be exploited for therapies that are safer or more effective than those in current use (1–5). For such therapies to be practical, they require three elements: a vulnerability in a cancer that is absent or very much reduced in normal cells, an agent that exploits the vulnerability, and a reliable biomarker for identifying which tumors are likely to respond to the therapeutic agent. As knowledge of the molecular changes in cancer has increased, it has become possible to generate hypotheses about specific vulnerabilities that might be linked to the genetic changes that support the growth of cancer cells (6–9) or that defeat the strategies that cancer cells use to avoid apoptosis (10–12). This approach has the benefit that it starts with a known molecular change that can serve as a biomarker for vulnerability, but it is limited by the fact that there is much about the biology of cancer that

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we do not understand (6). An alternative approach logically similar to classical forward genetics is to use phenotypic screening to identify agents that selectively kill cancer cells. This approach has the advantage of being unbiased and not limited by our knowledge of cancer biology and the disadvantage that identifying the biologically relevant target(s) of an interesting small molecule is quite difficult.

In an unbiased approach to identify cancer-selective vulnerabilities in non-small cell lung cancer (NSCLC), we screened a well-annotated panel of NSCLC cell lines (13) with small, drug-like (14) synthetic chemicals to identify compounds that killed some, but not all of the cancer cell lines and did not kill immortalized human bronchial epithelial cell (HBEC) lines (4). In this way we sought to demonstrate selective toxicity as a starting point for identifying compounds of interest. The cell panel that we used has exome sequencing, gene expression, siRNA and drug sensitivity profiles, providing opportunity for discovery of biomarkers associated with any vulnerability to a chemical. NSCLC cell lines are particularly useful as they have many more mutations than most other cancer cell types (15) and thus may contain a wide selection of mutation-derived vulnerabilities associated with cancer. From the compounds that fulfilled our screening criteria, we selected indolotriazine SW044248 for an investigation of its mechanism of action because it killed approximately 15% of 74 NSCLC cell lines and was not toxic at all to three HBEC lines, suggesting that this compound exploited a vulnerability that was fairly common in NSCLC but not in normal lung-derived cells. In addition, SW044248 killed HCC4017 cells but not HBEC30KT cells which were derived from the same patient, allowing us to compare cancer with non-cancer cells that originally shared the same genetic background. SW044248 induced rapid, complete inhibition of macromolecular synthesis in sensitive cells, induced the integrated stress response, and inhibited purified topoisomerase I (Top1) *in vitro*.

Top1 inhibitors are used clinically in cancer therapy. Most of these function by binding to both Top1 and DNA(16, 17). Top1 relaxes DNA supercoils through an attack on the phosphodiester bond of one strand forming a transient covalent link between the enzyme and DNA(18). The cleaved strand rotates once and reforms the phosphodiester bond, releasing Top1. The Top1 inhibitors in clinical use function by binding the interface between Top1 and the DNA, preventing full strand rotation after cleavage and leaving Top1 covalently linked to DNA (19). Repair mechanisms can remove Top1 from the DNA by proteolysis, but during S phase this process is not efficient enough to prevent DNA polymerases from colliding with Top1 adducts. This causes replication forks to collapse and generates DNA double strand breaks. As a consequence, the Top1 inhibitors in clinical use are generally toxic for rapidly growing tissues, such as the intestinal epithelium. There is another class of “non-canonical” Top1 inhibitors that do not appear to cause covalent links between Top1 and DNA, but these have not been explored extensively for therapy (20–22). However, Top1 has many activities in cells in addition to unwinding DNA supercoils and these activities might be exploited therapeutically by inhibitors that do not primarily kill cells through replication-coupled DNA damage (23). Here we report that SW044248 is a novel non-canonical Top1 inhibitor with a pattern of selective toxicity for NSCLC cells quite different from the canonical interfacial Top1 inhibitor camptothecin.

Materials and Methods

Cell Culture

All the immortalized HBEC and NSCLC cell lines were obtained from John D. Minna at UT Southwestern Medical Center. All cell lines except HBEC30KT were cultured in RPMI 1640 media supplemented with 5% fetal bovine serum (FBS) (Atlanta Biologicals Cat # S11195) and 1% HEPES (Life Technologies, Cat # 15630080). HBEC30KT cells (24) were cultured in Epi-CM media (ScienceCell Research Labs, Cat # 4101) supplemented with 2% FBS, 1% Epi CGS media and 1% HEPES. The cells were cultured at 37 C in a 5% CO₂ incubator. The identities of all cell lines were confirmed by short tandem repeat (STR) analysis of cellular DNA (PowerPlex 1.2 Kit, Promega Corp, Madison, WI) at the time cells were taken out of liquid nitrogen for use and all were tested and found to be free of mycoplasma (e-Myco Kit, Boca Scientific).

Small Molecules

SW044248 (ChemDiv Cat # 5471-0032), SW202742 (ChemBridge Cat #7411554), Camptothecin (Sigma Cat # C9911), Etoposide (Sigma Cat # E1383), Cisplatin (Sigma Cat # P4394), Actinomycin-D (Sigma Cat # A1410) and cyclohexamide (Sigma Cat # C7698) were purchased commercially. The chemicals were all dissolved to 10 mM concentrations in DMSO and stored at -20°C. All the cell treatments were performed by dissolving the compounds in the appropriate cell culture media (RPMI 1640 or EpiCM) by vortexing in sterile 15 ml tubes.

High Throughput Screening

High throughput screening of The UT Southwestern compound library on the NSCLC and HBEC cell lines was performed as described earlier (4).

Cell ATP Assay

100 µL of 50,000 cells/ml cell suspensions of individual cell lines were added in wells in 96-well plates. The next day, 100 µL of cell medium substituted with 2X concentration of SW044248 or camptothecin or DMSO in triplicates was added to each well. After 96 and 120 hours the ATP concentration in the wells was measured with CelTiter-Glo (Promega, Cat # G7573) following the manufacturer's protocol. The luminescence was measured with an Envision plate reader (Perkin Elmer).

Neutral Red Uptake Assay

For each cell line tested, 400 µL of a 50,000 cells/ml cell suspension was plated in wells in 48-well plates. The following day, 400 µL of cell medium substituted with 2X the final desired concentration of SW044248 or DMSO was added to each well. Concentrations were measured in triplicate. After 96 or 120 hours neutral red dye (Sigma Cat # N2889) was added to each well in a final concentration of 1% for 1 hour at 37 C. This media was aspirated and wells were washed twice with PBS. 500 µL of a solution of 50% ethanol, 1% glacial acetic acid in water was added to each well and plates were incubated on a Stovall Bellydancer shaker (Cole-Parmer cat # EW-51650), for 20 min. The extraction solution was

collected and the absorbance measured at 540 nm in a UV/Visual spectrophotometer (Ultrospec pro 2100, Amersham Biosciences).

SW044248 uptake assay and stability assays

HCC44 and HCC4017 cells were cultured in duplicate and assays of intracellular accumulation and compound stability were as described previously (4).

Western Blotting

To measure protein levels in response to various treatments (described in the Results section), the cell cultures were incubated on ice and the media aspirated. The cells were then washed with pre-chilled PBS at 4°C and then lysed in 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8 (RIPA buffer) with protease (Roche Cat # 11697498001) and phosphatase inhibitors (Sigma Cat # P5726, P0044). Protein concentrations were determined using Bradford's Reagent (Amresco Cat # M172) and cell lysates for each sample were diluted with 4X SDS sample buffer (200 mM Tris-Cl pH 6.8, 400 mM DTT, 8% SDS, 0.4% bromophenol blue, 40% glycerol), and boiled immediately for 10 minutes. The samples were stored at -80°C and 50 µg protein of each sample was loaded in 15- or 10-well 7.5–15% gradient acrylamide gels. The samples were resolved with SDS/PAGE. Proteins were electrotransferred to nitrocellulose membrane (Bio-Rad Cat # 162-0112) and probed with indicated primary antibody at 4°C overnight. All antibodies were diluted in antibody dilution buffer (5% fat-free milk powder in 1x PBS plus 0.2% TWEEN 20 (Sigma Cat # P2287)). All primary antibodies were used at a dilution of 1:1,000, with the exception of β-actin and topoisomerase-1, used at 1:10,000 and 1:5000 respectively. The primary antibodies used for the studies were: cleaved PARP (Cat # 9541), cleaved caspase-3 (Cat # 9661), β-actin (Cat # 3700), phospho eIF2α Ser 51 (Cat # 9721), PKR (Cat # 3072), ATF4 (Cat # 11815), CHOP (Cat # 5554), phospho ATM Ser 1981 (Cat # 13050), phospho Chk2 thr 68 (Cat # 2661), Chk2 (Cat # 2662), phospho ATR Ser 428 (Cat # 2853), phospho Chk1 Ser 345 (Cat # 2341), Chk1 (Cat # 2345), phospho P53 Ser 15 (Cat # 9284), P53 (Cat # 9282), phospho histone H2AX Ser 139 (Cat # 9718), p21^{CDKN1A} (Cat # 2947) (All Cell Signaling Technologies), phospho GCN2 Thr 899 (Cat # ab75836), GCN2 (Cat # ab137543), phospho PKR Thr 446 (Cat # ab32036) and Topo1 (Cat # ab109374) (All Abcam). Secondary HRP-conjugated goat anti-rabbit (BioRad Cat # 170-6515) and goat anti-mouse antibody (BioRad Cat # 172-1011) were diluted 1:10,000 in antibody dilution buffer. Protein bands were visualized using the ECL reagent (PerkinElmerLife) recorded on X-ray film and digital images collected with an Imagescan MTA 1100 flat bed scanner (Amersham).

All Western blots shown are representatives of at least three independent experiments performed on different days with independent sample sets.

EdU and EU Click-It DNA staining assay

50,000 cells were plated in a 12-well plate on a pre-autoclaved glass coverslip and incubated at 37°C for 48 hours. Cells were then treated with a series of concentrations of SW044248 or DMSO for 4.5 hours, followed by treatment with 10 µM EdU or EU (Life Technologies Cat # C10337 or Cat # C10330) and SW044248 for 1.5 hours. Cells were fixed with 3.7%

formaldehyde in PBS for 20 min and Alexa dyes were conjugated to the alkyne-labeled EDu or EU by Click chemistry following the manufacturer's instructions. Coverslips were mounted on slides in DAPI-containing mounting media (Sigma Cat # F6057). The cells were visualized and digital images collected with an EVOS-FL Cell Imaging System microscope (Life Technologies Cat # AMF4300) with a GFP and UV filter or Texas Red and UV filter.

HPG Click-It Protein Synthesis assay

Cells were plated and incubated as described above. Cells were treated with a series of concentrations of SW044248 or DMSO for 4.5 hours, followed by treatment with 50 μ M HPG in L-methionine-free RPMI-1640 media and SW044248 for 1.5 hours. Cells were processed as described above following manufacturer's protocol (Life Technologies Cat # C10428). Samples were visualized and photographed as described above using Texas Red and UV filters.

RNA-Seq

HCC4017 cells were plated into 15 cm tissue culture dishes for 24 hours until they reached ~70–80% confluency. Duplicate samples were treated for 6 hours with 2 μ M of SW044248 or DMSO as the control. DNA-free RNA was isolated using TRIzol Reagent (Life Technologies Cat # 15596018) with the PureLink RNA Mini Kit (Invitrogen Cat # 12183018A) according to manufactures instructions, including on-column PureLink DNase (Life Technologies Cat # 12185010) treatment. The yield and quality of total RNA (RNA Integrity Number - RIN) was analyzed using the Bioanalyzer NanoChip service provided by UT Southwestern Genomics and Microarray Core Facility. The RNA-seq library was generated from the total RNA and quantified on HiSeq2000 (Illumina) and the data analyzed by the McDermott Bioinformatics Core at UT Southwestern using TopHat and Cufflinks (25).

Tardis (Trapped in agarose DNA immunostaining) assay

HCC4017 cells were seeded overnight on glass coverslips placed in a multi-well plate at 4×10^5 cells/ml in 0.25 ml RPMI with 10% FBS. Following treatment with 10 μ M SW044248, 2.5 μ M Camptothecin or DMSO control for the indicated times, the coverslip was removed from the well and immersed in a solution of low melting point agarose from Sigma (0.5% w/v in PBS) to form a thin and evenly spread coating on the coverslip. The coverslips were placed into another multi-well plate on a cold surface (0°C) to solidify the agarose. The coverslips in the wells were then exposed to lysis buffer (1% w/v SDS, 80 mM phosphate buffer pH 6.8, 10 mM EDTA with protease inhibitor cocktail (Roche) and 1 mM DTT) for 30 min at 37°C then with 1 M NaCl supplemented with protease inhibitor cocktail and 1 mM DTT for 30 min at room temperature. After washing three times for 5 min each in PBS, the coverslips were exposed to primary antiserum overnight at 4°C. Monoclonal rabbit antibody to human topoisomerase I from Abcam was diluted 1/250 in PBS containing 0.1% v/v Tween 20 (PBST) and 1% w/v BSA. The coverslips were washed for 5 min three times in PBST. The slides were then exposed to Alexa Fluor 488 goat anti-rabbit secondary antibody for 2 hours at room temperature, diluted 1 in 100 in PBST containing 1% w/v BSA followed by three 5 min washes in PBST. Coverslips were stained with Hoechst 33342 dye

(10 μ M in PBS) for 5 min and imaged at 10X magnification with an EVOS-FL Cell Imaging System microscope (Life Technologies) with GFP and UV filters.

Ingenuity Pathways Analysis

For each gene, expression levels of duplicate samples were averaged and genes for which the sum of average RPKM with and without treatment with compound were less than 1.0 were removed from the data set (low expressed). The log₂ of the ratio of expression with compound and without was calculated and genes with values between log₂= 0.4 and log₂ = -0.4 were removed from the list (changes of ~ less than 24%). A false discovery rate was calculated (26) and genes with FDR>.49 were removed. The remaining list of 834 genes with their log₂ values for expression change in the presence of SW044248 was analyzed with the Ingenuity Pathways Upstream Analysis option.

siRNA Transfections

Individual siRNAs for PKR (Cat # S102223018, S102223011), PERK (Cat # S102223718, S102223725), ATF4 (Cat # S103019345), CHOP (Cat # S100059535, S103041633) and Top1 (Cat # S100050274, S102662366) and negative control (Cat # 1027281) were purchased from Qiagen. GCN2 (ID #J-005314-05, J-005314-06, J-005314-07, J-005314-08) and p21 (ID # J-003471-11, J-003471-12) siRNAs were purchased from Dharmacon. For siRNA transfections, 100 μ l of Opti-MEM (Life Technologies Cat # 51985091) was added to a sterile microfuge tube. 2 μ l of Lipofectamine RNAi-MAX transfection reagent (Life Technologies Cat # 13778150) was added to the tube, mixed by pipetting and incubated for 10 minutes. 1.2 μ l of 5 μ M siRNA stock was added to the tube and incubated for 10 min after thorough mixing. 500 μ l of cell culture medium containing 50,000 cells was added to the tube and mixed gently. The contents of the tube were transferred to a well of a 12-well plate. For cell-survival experiments, 60 μ L of the cell and siRNA solution was added to each well of a 96-well plate. The cells were allowed to grow for 72 hours. For western blot experiments, the cell culture medium was aspirated, wells were washed with 1ml PBS followed by treatment with fresh cell culture medium substituted with SW044248 or DMSO. For cell viability experiments, 60 μ l of 10 nM siRNA in Opti-MEM + RPMI 1640 media was added to each well with the desired final concentration of SW044248 or DMSO vehicle for controls. The plates were further incubated for appropriate times for assay of cell viability.

Assay of relaxation of supercoiled DNA by Top1

The manufacturer's protocol for the Topoisomerase-1 Assay Kit from Topogen (Cat # TG1015) was followed with slight modifications. The assay was designed to test the ability of a test compound to interfere with the DNA uncoiling activity of 1 unit Topo1. For each reaction 20 μ l of reaction mix was added to a thin-walled 200 μ l PCR tube and incubated in ice prior to addition of compounds. The reaction mix consisted of 2 μ l 10X TGS Buffer, 16.9 μ l RNAase-free water, 1 μ l of 250 ng/ μ L supercoiled DNA and 0.1 μ l of 10U/ μ l Topo1 enzyme. One tube was designated as supercoiled DNA control and contained all components except Topo1 enzyme. For test samples 2 μ l of test compound was added to the reaction mix and incubated at 37°C for 30 minutes. After the incubation, 4 μ l of 5X stop buffer was added to each tube and followed by addition of 25 μ l 24:1 chloroform: isoamyl alcohol solution

(Sigma Cat # C0549) with mixing by pipette. The tubes were centrifuged in a microfuge for 15 seconds and the 20 μ l the blueaqueous layer containing DNA was loaded on a 1.5% agarose gel (in TPE buffer) containing 0.2 μ g/ml chloroquine. The gel was run in TPE buffer containing 0.2 μ g/ml chloroquine at 45 V for 5 hours for optimum separation of bands. The gel was stained in 0.25 μ g/ml ethidium bromide solution for 15 min and destained in distilled water for 10 min. The gel was visualized with a UV gel documentation system (Alpha Innotech).

Topoisomerase-2 (Topo2) DNA decatenation assay

We purchased the commercial Topoisomerase-2 Assay Kit from Topogen (Cat # TG1101) and followed the manufacturer's protocol to determine the effect of test compounds on Topo2-mediated DNA decatenation.

Plasmid construction and isolation of p21 stable cell line

p21^{CDNK1A} cDNA was purchased from OriGene Technologies, Inc (Rockville, MD). The cDNA fragment was amplified by PCR using the forward primer GATCAGAATTCCCACCATGTCAGAACCGGCT and the reverse primer GATCACTCGAGTTAGGGCTTCCTCTTGGA. The amplified fragment was inserted between the EcoRI and XhoI sites of the pCMV-Script Vector (Stratagene, La Jolla, CA). Following transformation into competent cells (One Shot Max Efficiency DH5alpha T1, Invitrogen, Carlsbad, CA) the plasmid was purified using the EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA) and the DNA sequence was verified. HCC4017 cells were transfected with p21-pCMV-Script plasmid using Lipofectamine LTX and Plus Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Stable single clones surviving selection with 1.2 mg/ml of G418 for two weeks were isolated with cloning cylinders.

Statistical Analysis

Pair-wise comparisons were performed with a two tailed student's T-test.

RESULTS

SW044248 is selectively toxic for some NSCLC cell lines

In a screen of the UT Southwestern chemical compound library against a panel of 74 non-small lung cancer (NSCLC) cell lines, compounds were selected that killed cells of some, but not all, cancer lines and did not kill immortalized lung epithelial cells (4). One of these, indolotriazine SW044248, was selectively toxic for approximately 15% of the NSCLC cell lines (Figure 1A). An orthogonal cell viability assay confirmed that HCC4017, H292 and H1819 were sensitive to SW044248 whereas five other NSCLC cell lines and immortalized human bronchial epithelial cell line HBEC30KT were relatively resistant (Figure 1B). To determine if SW044248 was cytotoxic or cytostatic, HCC4017 and HBEC30KT cells that had been incubated overnight with 2, 6 and 10 μ M SW044248 were analyzed by western blotting for cleaved poly-(ADP-ribose) polymerase 1 (PARP) and cleaved caspase-3 as markers of apoptosis. SW044248 caused cleavage of PARP and caspase-3 in a concentration-dependent manner in HCC4017 cells but not in HBEC30KT cells (Figure 1C)

as early as 4 hours of treatment in HCC4017 (Figure 1D). SW044248 also induced PARP cleavage in sensitive H292 and H1819 cells but not in resistant NSCLC lines HCC44 and H2122 (Figure S1A). HBEC30KT cells (24) were cultured in a different growth medium than the cancer cells; however, no significant difference in SW044248 toxicity was observed in HCC4017 cells cultured in either cancer cell or epithelial growth medium (Figure S1B). The intracellular concentration of SW044248 and its chemical stability did not differ significantly between sensitive (HCC4017) or resistant cell lines (HCC44, HCC4017 resistant clones R7 and R17) (Figure S1C) or HBEC30KT cells (Figure S1D). Taken together, these data suggested that SW044248 is a selectively cytotoxic chemical with activity against certain NSCLC cell lines.

In our chemical library we had an analog to SW044248, SW202742, which has an isopropyl group instead of ethyl at the indole nitrogen (Figure 1E). HCC4017 cells were relatively insensitive to SW202742 (Figure 1F). SW202742 was taken up by HCC4017 cells similar to SW044248 (Figure S1D). This provided us with a negative control compound very similar to SW044248 in chemical properties.

SW044248 rapidly inhibits transcription, translation and DNA synthesis in sensitive cells but not insensitive cells

To investigate the mechanism of action of SW044248, we determined the effect of the compound on DNA replication, global RNA synthesis and protein synthesis. HCC4017 and HBEC30KT cells were treated with increasing concentrations of SW044248 for 5 hours followed by labeling for an additional 45 minutes with alkyne-modified nucleotide analogs, 5-ethynyl-2'-deoxyuridine (EdU) for studying DNA replication, 5-ethynyl-2'-uridine (EU) for RNA transcription or amino acid homopropargylglycine (HPG) for studying protein translation. The cells were fixed and processed to add Alexa-labeled azide dyes to the alkyne-modified macromolecules and the cells were imaged by fluorescence microscopy. SW044248 rapidly blocked DNA replication, transcription of RNA and protein translation in HCC4017 cells (Figure 2A) and H292 cells (Figure S2A) but not in resistant HBEC30KT cells (Figure 2B) or HCC44 cells (Figure S2A). In contrast, 6 h treatment with 10 μ M of the inactive compound SW202742 did not inhibit macromolecular synthesis in HCC4017 cells (Figure S2B). These observations indicate that SW044248 inhibits multiple crucial cell processes required for survival.

The acute transcriptional response to SW044248 correlates with the response to agents that cause DNA damage

A traditional approach to identifying the mechanism of action of an unknown compound is to compare its biological activity to those of agents with known mechanisms of action (27, 28). One way to do this is to investigate the transcriptional response to a new agent and correlate this to the transcriptional responses of known agents. To study the acute cellular response to SW044248, gene-expression in HCC4017 and HBEC30KT cells treated with 2 μ M SW044248 for 6 h was measured by sequencing mRNA. There was essentially no change in transcription in HBEC30KT cells treated with the compound and a robust response in HCC4017 cells. To identify candidates for the type of stress caused by SW044248, we used IPA Upstream Analysis to predict which chemical agents with known

targets or modes of action would produce a transcriptional response significantly correlated with the response to SW044248. Almost half of the agents predicted by IPA to be most correlated were chemicals that can induce DNA damage, with inhibitors of topoisomerase I (Top1) or II (Top2) having very high scores predicting activity (Table S1).

To determine if this transcriptional response was accompanied by changes in activity of proteins involved in the response to DNA damage, cells were probed by immunoblotting. Several proteins that are known to either sense DNA damage, ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related protein (ATR), or known to be phosphorylated following DNA damage, histone H2AX, CHK2, CHK1 and P53, were found to be activated in HCC4017 (Figure S3A) but not in HCC44 cells (Figure S3B) following SW044248 treatment. HCC4017 cells also had elevated DNA damage signaling compared to HCC44 cells in the absence of treatment with SW044248 (Figure S3A).

To determine if SW044248 inhibited Top2, a target of doxorubicin, the agent predicted to be most correlated to SW044248, the compound was added to an *in vitro* assay of the ability of purified Top2 to decatenate DNA plasmids (Figure 3A). SW044248 and the Top1 inhibitor camptothecin (CPT) were unable to inhibit Top2, whereas the Top2 inhibitors etoposide, cisplatin, and the non-specific DNA intercalator actinomycin (not shown) did inhibit the assay. Thus, SW044248 was not a Top2 inhibitor or a DNA intercalator. However, SW044248 did inhibit the ability of purified Top1 to convert supercoiled DNA into relaxed topoisomers and open circle DNA (Figure 3B) and this activity directly correlated with compound concentration (Figure 3C). The non-toxic analog SW202742 did not block Top1-induced relaxation of supercoiled DNA (Figure 3D), suggesting that the two activities of SW044248, inhibition of Top1 and induction of cell death by apoptosis, might be related.

The cellular response to SW044248 differs from the response to CPT

The Top1 inhibitor CPT did not show the differential cytotoxicity observed with SW044248, as HCC4017 and non-cancer HBEC30KT cells were equally sensitive to CPT (Figure S4A). We noticed that as the amount of Top1 enzyme in the *in vitro* assays of Top1 activity increased, SW044248 and CPT did not produce identical effects (Figure S4B). CPT causes Top1 to become covalently linked to the DNA at the site where it creates a single stranded break (16). Thus, as the amount of Top1 increases in the presence of CPT it converts supercoiled DNA into a series of topoisomers that run slower on gel electrophoresis than the relaxed topoisomers generated by Top1 alone (Figure S4B). In the same type of assay, SW044248 inhibition of Top1 preserved the supercoiled DNA and generated few relaxed topoisomers. This suggested that the inhibition of Top1 by SW044248 might not result in nicking the DNA followed by a covalent link to the proteins. If so, with the proper stoichiometry and/or timing, SW044248 might prevent CPT from forming relaxed topoisomers in the *in vitro* assay. When present in two-fold excess, SW044248 did prevent CPT from converting supercoiled DNA into relaxed topoisomers (Figure 3E). In cells, covalent linkage of Top1 to DNA by CPT is followed by degradation of Top1 (29). Treating HCC4017 cells with either CPT or SW044248 for 3 or 6 hours resulted in degradation of Top1 in the CPT treated cells, but not the SW044248 treated cells (Figure 3F). However, when HCC4017 cells were treated with 1% DMSO (control) or SW044248 for 3 hours and

then CPT was added, CPT-induced degradation of Top1 was blocked in the samples containing SW044248 (Figure 3G). The non-toxic compound SW202742 could not prevent the degradation of Top1 induced by CPT in either HCC4017 or H292 cells (Figure S4C,D). Thus, SW044248 appeared to inhibit Top1 by a mechanism different from CPT. An assay used for the detection of covalent linkage of Top1 to DNA by CPT, the TARDIS assay (30, 31), involves treating cells with an agent such as CPT, embedding the cells in agarose and lysing them under conditions that allow the denatured proteins to diffuse out of the agarose leaving those covalently linked to DNA trapped in the agarose. These proteins, such as Top1, can then be detected by immunofluorescence. When HCC4017 cells treated with 2.5 μ M CPT or 10 μ M SW044248 for an hour were analyzed by TARDIS, CPT caused Top1 to be retained in the agarose and SW044248 did not (Figure 3H). Since SW044248, unlike CPT, did not induce the proteolysis of Top1, we treated HCC4017 cells longer, for 6h, before examining cells by TARDIS (Figure S4E). Some Top1 was retained in the agarose under these conditions, although the fluorescent signal was reduced compared to 1 h treatment with CPT (Figure S4E). Thus, Top1 inhibition by SW044248 can cause covalent trapping of the enzyme on DNA, but with kinetics far slower or to an extent much less than with CPT.

In addition to correlating to the effects CPT, the acute transcriptional response to SW044248 included upregulation of many genes that are targets of the transcription factor ATF4. Upstream Analysis with IPA software predicted activation of three of four kinases that drive this response (32, 33), General Control Nonderepressible 2 (GCN2 or EIF2AK4), PKR-Like Endoplasmic Reticulum Kinase (PERK or EIF2AK3) and Protein Kinase RNA-Activated (PKR or EIF2AK2) (Table 1). Upon activation, these kinases phosphorylate and inactivate eukaryotic Initiation Factor 2 Alpha (eIF2 α), inhibiting protein translation that depends upon eIF2 but facilitating translation of the transcription factor ATF4 (33) (Figure 4A). When cells sensitive or resistant to SW044248 were treated with 2 μ M compound for 6 hours, eIF2 α phosphorylation increased in the sensitive but not the resistant cell lines (Figure 4B). SW044248 induced phosphorylation of GCN2, PKR and eIF2 α as early as 2 hours after addition of compound and increased expression of the ATF4 protein (Figures 4C and S5A) and its pro-apoptotic client CHOP (DNA Damage-Inducible Transcript 3, DDIT3) (34, 35) in sensitive HCC4017 (Figure 4C) and sensitive H292 cells. SW044248 did not cause phosphorylation of eIF2 α in HBEC30KT cells or in HCC4017 clone 7 which were resistant to the compound (Figure S5B). Inactive SW202742 also did not induce the phosphorylation of eIF2 α in any of the cell lines tested (Figure S5B). CPT induced phosphorylation of GCN2 similar to SW044248 but was much less effective for activation of PKR (Figure 4D). In cells treated with CPT, eIF2 α was less phosphorylated and the target of ATF4, CHOP, was only weakly activated compared to cells treated with SW044248. Phosphorylation of eIF2 α induced by SW044248 required its activity on Top1. HCC4017 were either pretreated for 2 h with 5 μ M CPT to begin the degradation of Top1 (Figure 4E) or were pretreated for 2h with 10 μ M SW044248 (Figure 4F). Then SW044248 was added to half the samples in the presence of CPT pretreatment (Figure 4E) or 5 μ M CPT was added to half the samples in the presence of SW044248 (Figure 4GF) for a chase period of up to 20 h. When Top1 levels were lowered by CPT pretreatment, phosphorylation of eIF2 α in samples treated with SW044248 was reduced compared to without CPT. This is most

evident comparing the 20 h chase time (compare panel E to panel F). In the absence of CPT, SW044248 increased phosphorylation of eIF2 α over time compared when CPT was present, whereas when CPT was present the phosphorylation of eIF2 α decreased over time in parallel with reduction in Top1. Taken together, all these data suggest that SW044248 can activate the integrated stress response by activating several stress-sensor kinases in sensitive cell lines leading to inhibition of protein translation. This response is reduced when Top1 is degraded after treatment with CPT.

Since SW044248 induced apoptosis (Figure 1C and 1D) and also the integrated stress response (Figure 4), the SW044248-induced integrated stress response might play a role in its toxicity in HCC4017 cells. To test this hypothesis we eliminated expression of elements of the integrated stress response by siRNA and measured the effect on the apoptotic response to SW044248 in HCC4017 cells. Eliminating ATF4 or CHOP, PERK or PKR (Figure S6) had no effect on PARP cleavage induced by SW044248 or its toxicity. However, eliminating GCN2 reduced phosphorylation of PKR, cleavage of PARP and the toxicity of SW044248 (Figure 5A,C).

The inhibition of Top1 in HCC4017 cells contributes to the toxicity of SW044248

To determine if the inhibition of Top1 by SW044248 played a role in its toxicity, we depleted Top1 by siRNA and treated cells with the compound and measured the responses we have described previously. Top1 depletion in HCC4017 cells with two individual siRNAs reduced PARP cleavage, the phosphorylation of GCN2 and eIF2 α , and prevented the increase of ATF4 in response to SW044248. Top1 knockdown also decreased phosphorylation of CHK2 in cells treated with SW044248 (Figure 5B). Thus, inhibition of Top1 by SW044248 plays a role in the induction of an integrated stress response and of apoptosis in HCC4017 cells. The siRNA-mediated Top1 knockdown also significantly, but incompletely rescued HCC4017 cells from SW044248 toxicity (Figure 5D).

HBEC30KT and HCC44 cell lines protect themselves from SW044248 by up-regulating p21^{CDKN1A}

In the course of investigating the protein response to DNA damage induced by SW044248, we noticed that although there was no acute change in transcription in non-cancer HBEC30KT or NSCLC HCC44 in response to SW044248, HBEC30KT (Figure 6A) and HCC44 (not shown) increased p21^{CDKN1A} protein in response to the compound. Inactive compound SW202742 did not increase p21^{CDKN1A} protein in HBEC30KT cells (Figure S5C). In contrast, in HCC4017 cells p21^{CDKN1A} was modified to a form that migrated faster on PAGE in response to lower concentrations of SW044248 and at higher concentrations was absent entirely (Figure 6A). Cleavage of the C-terminus of p21 has been reported to prevent it from binding to PCNA and participating in DNA repair processes (36–38). To determine if the p21^{CDKN1A} response in HBEC30KT or HCC44 cells played a role in their sensitivity to SW044248, we treated these cell lines with siRNA to knock down p21^{CDKN1A} expression and then treated them with 5 or 10 μ M SW044248 or the DMSO vehicle (Figure 6B,C). Both cell lines were sensitized to SW044248 when p21^{CDKN1A} was suppressed, although the cancer cell line was less affected than the HBEC cell line. We transfected HCC4017 with a plasmid expressing p21^{CDKN1A} cDNA and isolated clones

stably expressing p21^{CDKN1A} at levels comparable to expression in HBEC30KT (Figure 6D). Compared to the parental HCC4017 cells, HCC4017 cells stably expressing p21^{CDKN1A} had less phosphorylation of eIF2 α and H2AX and less toxicity in response to SW044248 (Figure 6E,F). Thus, at least part of the differential toxicity to SW044248 is due to the inability of sensitive cells to mount a protective response involving p21^{CDKN1A}.

Discussion

In a screen for chemicals that are selectively toxic for NSCLC lines, we identified a compound, SW044248, that rapidly inhibited macromolecular synthesis in sensitive cell lines and had no effect on other NSCLC lines or non-cancer HBEC cells (4). SW044248 was able to accumulate in the insensitive cells, thus the selectivity of the compounds was not due to uptake or efflux differences. By comparing the acute transcriptional response to SW044248 in sensitive HCC4017 cells to those of known agents, we found a significant correlation with inhibitors of topoisomerases. In *in vitro* assays, SW044248 inhibited Top1 and not Top2 and appeared to do so by a mechanism different from CPT. CPT and its synthetic derivatives inhibit Top1 by preventing the religation of the cleaved DNA single strand, leaving the enzyme trapped in covalent linkage to the DNA (16, 39). In *in vitro* assays, CPT allows Top1 to relax supercoiled DNA into a series of protein-linked topoisomers. In contrast, SW044248 prevented the enzyme from relaxing supercoiled DNA and, when present in excess, prevented CPT from generating topoisomers *in vitro*. In cells, CPT causes Top1 to cross-link to DNA and this can be visualized by immunofluorescence of unfixed cells embedded in agarose and lysed with detergent, which traps the DNA and any cross-linked proteins in place but allows unlinked proteins to diffuse away. After one hour treatment with CPT or SW044248, CPT trapped Top1 on the DNA and SW044248 did not. However, 6 h treatment with SW044248 did result in detectable cross-linking of Top1 to DNA, although less than in cells treated for one hour with CPT. In cells treated with CPT, Top1 trapped on DNA is removed by being degraded (29). In cells treated with SW044248, Top1 was not degraded and in cells treated with SW044248 and then CPT, Top1 was protected from the degradation induced by CPT. One possible explanation for these results would be that SW044248 competes with CPT for binding to Top1, and either prevents DNA nicking or has a greatly increased off rate, allowing relegation of DNA, as has been observed with some other Top1 inhibitors that differ in the degree to which they induce cross-linking to DNA (40).

The toxicity induced by SW044248 differed from that induced by CPT in several ways. CPT toxicity is mainly seen during S phase of the cell cycle, presumably due to the damage caused by DNA polymerases colliding with covalent Top1 adducts and inducing double strand breaks faster than repair mechanisms can remove cross-linked Top1 from the DNA (16). Although SW044248 induced a DNA damage response, the response to SW044248 was not limited to cells in S phase as transcription and translation were inhibited in all cells of a nonsynchronized cell population in 6 h, a period in which only 15% of the cells would be in S phase. SW044248 induced the integrated stress response faster and to a greater extent than did CPT, which is also consistent with the response to SW044248 not being limited to S phase.

Using siRNA knockdown, we showed that inhibition of Top1 contributed to the toxicity and cellular response of cells sensitive to SW044248. Another observation suggesting that inhibiting Top1 causes the selective toxicity of SW044248 is that the non-toxic compound SW202742, which differs from SW044248 by a single methyl group, also did not inhibit Top1 *in vitro*. However, because the protection in the siRNA experiments was incomplete, we do not eliminate the possibility that SW044248 has other targets in sensitive cells that might contribute to the selective toxicity of this compound.

Cells resistant to SW044248 increase expression of p21^{CDKN1A} in response to the compound whereas in sensitive HCC4017 cells p21^{CDKN1A} appears to be truncated and degraded. In addition to its role as a regulator of the cell cycle, p21^{CDKN1A} also plays a more direct role in the DNA damage response (41). p21^{CDKN1A} binds to PARP-1 and PCNA through its C-terminal region and may serve to modulate the interaction between the other two proteins during base excision repair (41–43). Consistent with a role for DNA damage as a factor in sensitivity to SW044248, we observed an activation of proteins involved in DNA damage repair in response to the compound. In particular, UV radiation is one of the few known common activators of GCN2 and PKR, the two kinases responding to SW044248. UV radiation induces cyclobutane pyrimidine dimers that are removed by base excision repair. Down-regulating Top1 by siRNA inhibits repair of DNA lesions induced by UV (44). HCC4017 cells have chronically elevated activation of DNA repair proteins, suggesting that they might also suffer chronic DNA damage. These observations in combination with those we report here suggest that SW044248 may be selectively toxic for cancer cells that have chronic DNA damage requiring repair by mechanisms that involve Top1.

Because the knockdown of Top1 protected cells from SW044248 rather than copying the toxic phenotype of the compound, Top1 inhibited by SW044248 gains a toxic function. Top1 is involved in many aspects of transcription(23, 45–47), RNA processing (48–52), and DNA replication (39). To accomplish these tasks, Top1 interacts with several multiprotein complexes(53). A possible explanation for the selective toxicity we observe with SW044248 is that the inhibited Top1 is trapping other proteins in a dead-end complex and those proteins are required to sustain sensitive, but not resistant cells. If so, p21^{CDKN1A} might either alleviate the need for the dead-end complex, or prevent it from forming. Although we have published a transcriptional signature that was used to correctly identify NSCLC lines that were sensitive to SW044248 (4), it is likely that it will be necessary to understand the underlying cause of differential toxicity of SW044248 to identify a biomarker that will be practical for indicating vulnerability to agents like SW044248.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

SW044248 is selectively toxic for certain NSCLC cell lines. A. IC₅₀ values from 96 h incubation in SW044248 for 46 NSCLC cell lines and 4 immortalized HBEC lines. Cellular ATP content was measured by CellTiterGlo in 384 well format in duplicate. B. Dose-response to 6 day treatment with SW044248 for 8 NSCLC and a HBEC30KT line. Cell viability was measured by uptake of neutral red. C. SW044248 induces apoptosis in HCC4017 but not HBEC30KT. Cells were treated overnight with the concentrations shown. D. 2 μ M of SW044248 induces cleavage of PARP beginning after 2 h in HCC4017 cells. E. Structure of SW202742 which has an additional methyl group at the position indicated by the colored circle. F. SW202742 is much less active than SW044248. The dose response of HCC4017 to the two compounds is shown. Cell viability was measured by neutral red after 6 days.

Figure 2. SW044248 rapidly inhibits RNA, DNA and protein synthesis in HCC4017 but not HBEC30KT cells. A. HCC4017 cells were treated for 6 h with the concentrations of SW044248 shown and then labeled for 45 min with Click-IT[®] (Molecular Probes) reagents for metabolic labeling of RNA (red), DNA (green), or protein (red). Cells were counter stained with DAPI (blue) to label nuclei. B. HBEC30KT cells were treated with 10 μ M SW044248 for 6 h and labeled as in part A.

Figure 3.

SW044248 and CPT inhibit Top1 differentially. A. SW044248 does not inhibit Top2. Concatenated DNA was incubated with 1% DMSO, 10 μM SW044248, 100 μM CPT, 100 μM etoposide, 10 μM cisplatin, 10 μM cycloheximide and 4U Top2 and electrophoresed on an agarose gel. DNA decatenated by Top2 enters the gel but stays in the loading well when Top2 is inhibited. B. SW044248 inhibits relaxation of supercoiled (SC) DNA *in vitro*. Concentrations of reagents were 10 μM SW044248, 10 μM CPT, 100 μM etoposide, 10 μM cisplatin, 10 μM cycloheximide and 1 U Top1. C. Inhibition of Top1 by SW044248 increases with increasing concentration. D. Non-toxic compound SW202742 does not inhibit Top1 in vitro. Concentrations are μM. Data are representative of 3 or more experiments. E. 30 min pretreatment of 10 U Top1 with SW044248 blocks CPT-induced uncoiling of supercoiled (SC) DNA. SC DNA was incubated with the agents shown and electrophoresed on an agarose gel. CPT increases nicked open circle DNA levels in presence of 10 U Top1 while SW044248 prevents unwinding of SC DNA. SW044248 pretreatment blocks CPT activity *in vitro*. F. Treatment with 1 μM CPT but not 5 μM SW044248 decreases cellular Top1 protein in HCC4017 cells. G. Treatment of HCC4017 cells with 10 μM SW044248 from 1 h before addition of 5 μM CPT blocks the decrease in Top1 protein induced by CPT. On the figure the hours indicate the time of chase after adding CPT. H. HCC4017 cells were treated with DMSO, 2.5 μM Camptothecin, or 10 μM SW044248 for 1 h and then embedded in agarose and extracted with detergent in a TARDIS assay to detect Top1 crosslinked to DNA. DNA was stained with Hoechst (blue) and Top1 with antibody (green). All panels of H are the same magnification. The size bar = 400 μm. SC DNA, supercoiled DNA; OC DNA, open circle, relaxed DNA; CPT, camptothecin; SW, SW044248.

Figure 4.

SW044248 rapidly activates the integrated stress response through kinases GCN2 and PKR. CPT is much less effective in activating the integrated stress response. A. eIF2α is phosphorylated and inhibited by the four kinases shown. ATF4 translation is enhanced under these conditions. B. Three NSCLC cell lines resistant and 3 cell lines sensitive to SW044248 were treated $\pm$ 5 μ M SW044248 for 6h. Cell lysates were immunoblotted for eIF2α phosphorylation on Ser51. C. HCC4017 cells were treated with 2 μ M SW044248 for the intervals shown, harvested and cell lysates immunoblotted for the proteins shown. D. HCC4017 cells were treated with DMSO, 2 μ M SW044248, or 10 μ M CPT for 3 or 6 h and cell lysates were analyzed by immunoblotting for the protein shown. E. Cells were pretreated for 2 h with 5 μ M CPT and then 5 μ M CPT $\pm$ 10 μ M SW044248 for the times shown. Samples were immunoblotted for Top1 and Ser51 phosphorylated eIF2α. The amount of eIF2α phosphorylation is positively correlated with the amount of Top1 remaining. CPT, camptothecin. F. HCC4017 cells were treated for 2 h with 10 μ M SW044248, then with either 10 μ M SW044248 alone (–) or with 5 μ M CPT and 10 μ M SW044248 for the times shown.

Figure 5.

siRNA knockdown of GCN2 or Top1 decreases the stress responses to SW044248. A. HCC4017 cells were treated with control or individual GCN2 siRNAs for 72 h and then treated with 2 μ M SW044248 (+) or DMSO (–) for 6 h. Cell lysates were probed by immunoblotting with antibodies specific for the proteins shown. B. HCC4017 cells were treated with two different siRNAs targeting Top1 or with a control siRNA for 72 hours and then treated $\pm$ 2 μ M SW044248 for 6 hours. The cells treated with the non-targeting control siRNA received DMSO at the same final concentration as in the compound treated cells. Immunoblots for the proteins are representative of 3 experiments. C. The ATP concentration was measured in cell samples treated with control or GCN2 siRNAs for 72 h, then treated an additional 48 h with 2 μ M SW044248 and additional siRNA. D. ATP levels of cells treated as in part A, with Top1 or with a control siRNA for 72 hours and then treated $\pm$ 2 μ M SW044248 for 48 hours, were measured by Celltiter-Glo. Error bars are standard deviations of the means of 3 samples (data is representative of 4 independent experiments).

Figure 6.

Cells resistant to SW044248 increase p21^{CDKN1A} and cells sensitive to SW044248 do not.

A. HBE30KT and HCC4017 cells were treated overnight with the concentrations of SW044248 shown, and lysates were immunoblotted for p21^{CDKN1A} and actin. B&C. HBE30KT or HCC44 cells were treated with control or siRNAs to p21^{CDKN1A} for 48 h and then treated with DMSO or SW044248 for 48h. ATP was measured with and normalized to the control siRNA treated with DMSO. D. Immunoblot for p21^{CDKN1A} expression. E. HCC4017 and HCC4017 p21^{CDKN1A} Clone 5 stably expressing p21^{CDKN1A} were treated 24 h with the concentrations of SW044248 shown and the phosphorylation of eIF2α and H2AX measured by immunoblotting. F. HCC4017 and HCC4017 p21^{CDKN1A} clone 5 were treated for 4 days with the concentrations of SW044248 shown and ATP was measured as an indication of cell viability. Graphed data are the means of triplicate samples, with SEM. Data is representative of two or more experiments.

Table 1

Upstream analysis predicts activation of eIF2a kinases.

Upstream Regulator	Log Ratio mRNA Change	Molecular Type	Predicted Activation State	Activation z-score	p-value of Overlap
ATF4	0.925	transcription factor	Activated	4.586	4.20×10^{-39}
EIF2AK2		kinase	Activated	3.965	1.66×10^{-17}
EIF2AK3		kinase	Activated	3.431	7.41×10^{-28}
EIF2AK4		kinase	Activated	2.412	1.60×10^{-07}

Ingenuity Pathways Upstream Analysis of RNAseq data comparing gene expression in HCC4017 cell after $6 \text{ h} \pm 2 \text{ }\mu\text{M}$ SW044248 predicts activation of kinases that phosphorylate eIF2a.