Identification of genotype-selective antitumor agents using synthetic lethal chemical screening in engineered human tumor cells

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Summary

We used synthetic lethal high-throughput screening to interrogate 23,550 compounds for their ability to kill engineered tumorigenic cells but not their isogenic normal cell counterparts. We identified known and novel compounds with genotype-selective activity, including doxorubicin, daunorubicin, mitoxantrone, camptothecin, sangivamycin, echinomycin, bouvardin, NSC146109, and a novel compound that we named erastin. These compounds have increased activity in the presence of hTERT, the SV40 large and small T oncoproteins, the human papillomavirus type 16 (HPV) E6 and E7 oncoproteins, and oncogenic HRAS. We found that overexpressing hTERT and either E7 or LT increased expression of topoisomerase 2α and that overexpressing RAS^{V12} and ST both increased expression of topoisomerase 1 and sensitized cells to a nonapoptotic cell death process initiated by erastin.

Introduction

Molecularly targeted therapeutics represent a promising new approach to anticancer drug discovery (Shawver et al., 2002). Using this approach, small molecules are designed to inhibit directly the very oncogenic proteins that are mutated or overexpressed in specific tumor cell types. By targeting specific molecular defects found within tumor cells, this approach may ultimately yield therapies tailored to each tumor's genetic makeup. Two recent examples of successful molecularly targeted anticancer therapeutics are Gleevec (imatinib mesylate), an inhibitor of the breakpoint cluster region-abelsen kinase (BCR-ABL) oncoprotein found in Philadelphia chromosome-positive chronic myelogenous leukemia (Capdeville et al., 2002), and Herceptin (trastuzumab), a monoclonal antibody targeted against the HER2/NEU oncoprotein found in metastatic breast cancers (Mokbel and Hassanally, 2001).

A complementary strategy involves searching for genotype-selective antitumor agents that become lethal to tumor cells only in the presence of specific oncoproteins or in the absence of specific tumor suppressors. Such genotype-selective compounds might target oncoproteins directly or they might target other critical proteins involved in oncoprotein-linked signaling networks. Compounds that have been reported to display synthetic lethality include (1) the rapamycin analog CCI-779 in myeloma cells lacking PTEN (Shi et al., 2002), (2) Gleevec in BCR-ABL-transformed cells (Druker et al., 1996), and (3) a variety of less well-characterized compounds (Stockwell et al., 1999; Torrance et al., 2001).

Over the past several years, we and others have engineered a series of human tumor cells with defined genetic elements in order to identify those critical pathways whose disruption leads to a tumorigenic phenotype (Hahn et al., 1999, 2002; Lessnick et al., 2002). We postulated that these experimentally transformed cells would permit us to identify genotype-selective agents from both known and novel compound sources that exhibit synthetic lethality in the presence of specific cancer-related alleles. Compounds with genotype-selective lethality may serve as molecular probes of signaling networks present in tumor cells and might serve as leads for subsequent development of clinically effective drugs with a favorable therapeutic index.

The ability of genotype-selective compounds to serve as

SIGNIFICANCE

Identifying genetic alterations that increase the sensitivity of human cells to specific compounds may ultimately allow for mechanistic dissection of oncogenic signaling networks and tailoring chemotherapy to specific tumor types. We have developed a systematic process for discovering small molecules with increased activity in cells harboring specific genetic changes. Using this system, we discovered that several clinically used antitumor agents are more potent and more active in the presence of specific genetic elements. Moreover, we identified a novel compound that selectively kills cells expressing the Small T oncoprotein and oncogenic RAS. These genetically targeted small molecules may also serve as leads for development of anticancer drugs with a favorable therapeutic index.

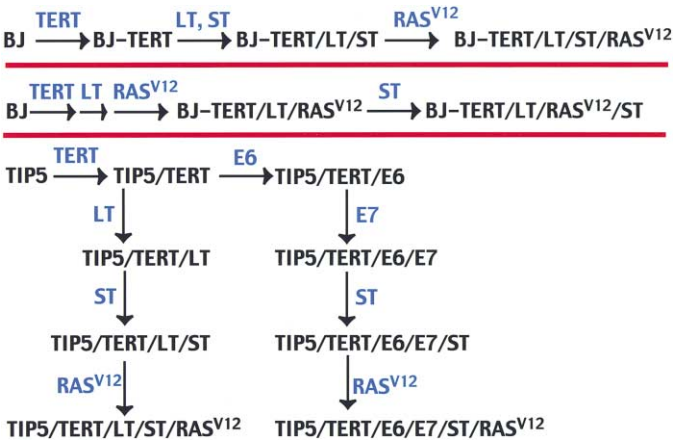

Figure 1. Experimentally transformed human cells

BJ cells are primary human foreskin fibroblasts. BJ-TERT cells are derived from BJ cells and express hTERT, the catalytic subunit of the enzyme telomerase. BJ-TERT/LT/ST cells are derived from BJ-TERT cells by introduction of a genomic construct encoding both simian virus 40 large (LT) and small T (ST) oncoproteins. BJ-TERT/LT/ST/RAS^{V12} tumor cells are derived from BJ-TERT/LT/ST cells by introduction of an oncogenic allele of *HRAS* (*RAS^{V12}*) (Hahn et al., 1999). BJ-TERT/LT/RAS^{V12} cells are derived from BJ cells by introduction of cDNA constructs encoding TERT, LT, RAS^{V12}, and a control vector (Hahn et al., 2002). BJ-TERT/LT/RAS^{V12}/ST cells are derived from BJ-TERT/LT/RAS^{V12} cells by introduction of a cDNA encoding ST (Hahn et al., 2002). TIP5 cells are primary human foreskin fibroblasts. The TIP5-derived cell lines were prepared by introducing vectors encoding hTERT, LT, ST, RAS, or the papillomavirus E6 or E7 proteins, as shown. E6 and E7 can jointly substitute for LT (Lessnick et al., 2002).

molecular probes is based on the premise of chemical genetics—that small molecules can be used to identify proteins and pathways underlying biological effects (Schreiber, 1998; Stockwell, 2000a, 2000b). For example, the observation that the natural product rapamycin retards cell growth made possible the discovery of the mammalian Target of Rapamycin (mTOR) as a protein that regulates cell growth (Brown et al., 1994; Sabatini et al., 1994). We have combined these two approaches, chemical and molecular genetic, to discover pathways affected by cancer-associated mutations.

Previous reports have indicated that it is possible to convert primary human cells into tumorigenic cells by the introduction of vectors expressing the hTERT and oncogenic RAS proteins as well as others that disrupt of the function of p53, RB, and PP2A (Hahn et al., 1999, 2002; Hahn and Weinberg, 2002; Lessnick et al., 2002). We made use of a series of engineered human tumorigenic cells and their precursors, which were created by introducing specific genetic elements into primary human foreskin fibroblasts (Figure 1). A variety of characteristics of these engineered tumorigenic cells have been reported previously, including their doubling time, their resistance to replicative senescence and crisis in culture, their response to γ irradiation, their ability to grow in an anchorage-independent fashion, and their ability to form tumors in immunodeficient mice (Hahn et al., 1999, 2002; Lessnick et al., 2002).

In one series of engineered cells, the following genetic elements were introduced sequentially into primary BJ fibroblasts: the human catalytic subunit of the enzyme telomerase (*hTERT*), a genomic construct encoding the Simian Virus 40 large (LT) and small T (ST) oncoproteins, and an oncogenic allele of *HRAS* (*RAS^{V12}*). In a second series, cell lines were created in which complementary DNA (cDNA) constructs encoding LT and ST were used in place of the SV40 genomic construct that encodes both of these viral proteins. In this latter series, ST was introduced in the last stage, enabling us to test compounds in the presence or absence of ST.

In a third series, we used cell lines derived from independently prepared human TIP5 foreskin fibroblasts created by introducing cDNA constructs encoding hTERT, LT, ST, and RAS^{V12} (Lessnick et al., 2002). In a fourth series, we used cell lines derived from TIP5 fibroblasts created by introducing cDNA constructs encoding hTERT, E6, E7, ST, and RAS^{V12}. In this series, HPV E6 and E7, which inactivate p53 and RB, respectively, serve a similar function as LT in the previous series. However, by using HPV E6 and E7, we were able to observe the effects of inactivating, separately and independently, p53 and RB. We report below the results of a large-scale screen for compounds that display selective killing of these engineered tumorigenic cell lines.

Results

We attempted to identify compounds with increased potency or activity in the presence of *hTERT*, *LT*, *ST*, *E6*, *E7*, or *RAS^{V12}*. Using engineered cell lines with these genetic elements, we screened 23,550 compounds, comprising 20,000 compounds from a combinatorial library, 1,990 compounds from the National Cancer Institute diversity collection, and 1,540 biologically active compounds that we had previously purchased and formatted into a screenable collection. In the primary screen, we tested in quadruplicate the effect of treating tumorigenic BJ-TERT/LT/ST/RAS^{V12} engineered tumorigenic cells with each compound for 48 hr at a concentration of 4 μg/ml, corresponding to 10 μM for a compound with a molecular weight of 400, which is the approximate median molecular weight of our libraries. We measured cell viability using the dye calcein acetoxymethyl ester (calcein AM) (Wang et al., 1993), which is a nonfluorescent compound that freely diffuses into cells. In live cells, calcein AM is cleaved by intracellular esterases, forming the anionic fluorescent derivative calcein, which cannot diffuse out of live cells. Hence, live cells exhibit a green fluorescence when incubated with calcein AM, whereas dead cells do not. Compounds that displayed 50% or greater inhibition of staining with the viability dye calcein AM in BJ-TERT/LT/ST/RAS^{V12} cells were subsequently tested in a 2-fold dilution series in BJ and BJ-TERT/LT/ST/RAS^{V12} cells to identify compounds that display synthetic lethality, i.e., lethality in tumorigenic cells but not in isogenic primary cells. We calculated the IC_{50} value (concentration required to inhibit 50% of the calcein AM signal) for each compound in each cell line (Table 1) and thereby identified nine compounds (Figure 2) that were at least 4-fold more potent in BJ-TERT/LT/ST/RAS^{V12} tumorigenic cells relative to BJ primary cells (i.e., compounds for which at least a 4-fold higher concentration was required in BJ primary cells in order to obtain the same 50% inhibition of calcein AM signal). We report below a more detailed analysis of these nine compounds.

Three of these compounds (doxorubicin, daunorubicin, and mitoxantrone) are in current clinical use as anticancer drugs, one (camptothecin) is a natural product analog of clinically used anticancer drugs (topotecan and irinotecan), and one (echino-

Table 1. Potencies of tumor-selective compounds in engineered cell lines

	BJ	BJ-TERT	BJ-TERT/LT/ST	BJ-TERT/LT/ST/Ras^{V12}	BJ-TERT/LT/Ras^{V12}	BJ-TERT/LT/ST	BJ-TERT/p53DD/CDK4^{R24C}/cyclinD1/ST/Ras^{V12}	TIP5-TERT	TIP5-TERT/LT/ST	TIP-TERT/E6	TIP5-TERT/E6E7	TIP5-TERT/E6E7/ST	TIP5-TERT/E6E7/ST/Ras^{V12}	Tumor selectivity	Genetic basis of selectivity
Echinomycin	>5	0.312	0.0048	0.0012	0.0048	0.0012	0.078	>5	5	0.0048	0.0024	>5	0.048	0.048	0.0048	>8333	nonspecific
Sangivamycin	0.312	0.039	0.195	0.078	0.078	0.078	0.078	1.25	0.312	0.039	0.078	0.156	0.078	0.078	0.078	4	nonspecific
NSC146109	>5	5	2.5	2.5	5	2.5	5	>5	>5	5	2.5	5	2.5	2.5	2.5	>4	nonspecific
Bouvardin	0.312	0.078	0.0195	0.078	0.078	0.0195	0.156	>5	0.312	0.039	0.039	0.078	0.039	0.039	0.078	4	nonspecific
Mitoxantrone	5	1.25	0.312	0.312	1.25	0.312	1.25	>5	1.25	0.625	1.25	1.25	0.625	0.625	1.25	16	TERT/RB
Doxorubicin	>5	1.25	0.312	1.25	1.25	1.25	1.25	>5	1.25	0.625	0.625	5	1.25	1.25	1.25	>8	TERT/RB
Daunorubicin	5	1.25	0.312	0.312	1.25	0.625	0.625	>5	1.25	0.625	0.625	5	0.625	0.625	0.625	16	TERT/RB
Camptothecin	>5	>5	1.25	0.0195	1.25	0.0195	1.25	>5	>5	0.156	0.156	>5	0.625	0.156	0.156	>512	RAS^{V12}/PP2A/RB
Erastin	>5	>5	>5	1.25	>5	1.25	2.5	>5	5	2.5	>5	>5	>5	5	5	>8	RAS^{V12}/PP2A

Nine tumor-selective compounds were retested in 16-point, two-fold dilution dose-curves in all engineered cell lines. The table lists the concentration (in μg/ml) required to achieve 50% inhibition of calcein AM staining (IC_{50}) for each compound in each cell line. The IC_{50} in primary BJ cells was divided by the IC_{50} in BJ-TERT/LT/ST/RAS^{V12} tumorigenic cells to obtain a tumor selectivity ratio for each compound. The compound selectivity for each genetic element was determined by calculating the selectivity ratio for each subsequent pair of cell lines in a series. Small T oncoprotein-selective compounds were considered to be selective for PP2A (the target of small T oncoprotein), whereas E6-selective compounds were considered to be selective for loss of p53, and E7-selective compounds were considered to be selective for loss of RB.

Figure 2. Chemical structures of nine genotype-selective compounds

mycin) was recently tested in phase II clinical trials. All nine compounds were subsequently tested in replicate at multiple doses in each panel of engineered cells to confirm that the observed selectivities were seen in multiple independently derived cell lines (Figure 1 and Table 1).

We developed a selectivity metric that measures the shift in the IC_{50} (concentration required for 50% inhibition of viability signal) of a compound in two different cell lines. To calculate this selectivity score between two cell lines, we divided the IC_{50} for a compound in one cell line by the IC_{50} for the same compound in a second cell line. Thus, a compound that must be used at a 4-fold higher concentration in one cell line relative to a second cell line would have a selectivity score of 4. We calculated the "tumor selectivity score" for each compound by dividing the IC_{50} value for the compound in the parental, primary BJ cells by the IC_{50} value for the compound in engineered BJ-TERT/LT/ST/RAS^{V12} cells, containing all four genetic elements required to create tumorigenic cells (Table 1).

These engineered tumorigenic cells make use of dominantly acting viral oncoproteins such as LT, ST, E6, and E7. These viral proteins are possibly involved in cell transformation in specific forms of cancer, namely simian virus 40-induced malignant mesothelioma (Testa and Giordano, 2001) and human papillomavirus-induced cervical carcinoma (Bosch et al., 2002), and have been used to disrupt p53 and pRB function to transform cells in vitro and in vivo (Elenbaas et al., 2001; Jorcyk et al., 1998; Perez-Stable et al., 1997; Rich et al., 2001; Sandmoller et al.,

1995). We made use of these two different methods for inactivating cellular proteins (i.e., we tested the effects of both LT and E6/E7-based inactivation of pRB and p53) in order to control for idiosyncratic effects that might be observed with a specific viral protein. We also confirmed the selectivity of these compounds in a cell line expressing dominant-negative inhibitors of p53 and pRB that are not derived from viral elements. This cell line expresses (1) a truncated form of p53 (p53DD) that disrupts tetramerization of endogenous p53, (2) a $CDK4^{R24C}$ mutant resistant to inhibition by $p16^{INK4A}$ and $p15^{INK4B}$ (the major negative regulators of CDK4), and (3) cyclin D1. We tested the effects of the nine genotype-selective compounds at a range of concentrations in these cells, which we refer to as BJ-TERT/p53DD/$CDK4^{R24C}$/D1/ST/RAS^{V12} cells (Table 1). We found that there was an overall modest reduction in activity for all of the compounds when tested in these cells, but that the overall results of our analysis were unchanged by the use of nonviral proteins in this cell line (Table 1).

We sought to determine the genetic basis of selectivity for each compound. That is, for each compound, we attempted to define the gene or combination of genes responsible for rendering cells sensitive to the compound (Table 1). We found that these nine compounds could be categorized into three groups, namely (1) compounds that displayed no simple genetic selectivity, (2) compounds that displayed selectivity for cells harboring TERT and inactive RB, and (3) compounds that required the presence of both oncogenic RAS and ST in order to exhibit lethality.

The compounds in group 1, sangivamycin, bouvardin, NSC146109, and echinomycin, have no clear genetic basis for their tumorigenic cell selectivity. For example, echinomycin becomes somewhat more active as each genetic element is introduced (Figure 3A). As we have observed that the rate of cell proliferation increases when each of these genetic elements is introduced, it is likely that the compounds in group 1 are simply selective for rapidly dividing cells. Supporting this interpretation is the fact that all of these compounds are reported to act by inhibiting DNA or protein synthesis, the need for which is greater in rapidly dividing cells. For example, echinomycin is reported to function as a DNA bis-intercalator (Van Dyke and Dervan, 1984; Waring and Wakelin, 1974), bouvardin is reported to function as a protein synthesis inhibitor (Zalacain et al., 1982), sangivamycin is a nucleotide analog (Rao, 1968), and NSC146109 structurally resembles a DNA intercalator (Figure 2). It should be noted that sangivamycin has been reported to function as a PKC inhibitor (Loomis and Bell, 1988), although this activity seems unlikely to be relevant in this context because other PKC inhibitors displayed no selectivity in this system (data not shown). We were able to identify and discard compounds that are simply more active in rapidly dividing cells, such as these group 1 compounds, because they show no clear genetic basis of selectivity. Thus, we were able to focus our mechanistic studies on the more interesting compounds in groups 2 and 3.

The compounds in group 2, mitoxantrone, doxorubicin, and daunorubicin, are topoisomerase II poisons, which bind to topoisomerase II and DNA and prevent the religation of double-strand DNA breaks introduced by topoisomerase II. These compounds, and anthracyclines in general, have also been reported to induce the formation of reactive oxygen species (ROS) in some cell types (Laurent and Jaffrezou, 2001; Muller et al., 1998; Richard et al., 2002), although we did not observe the formation

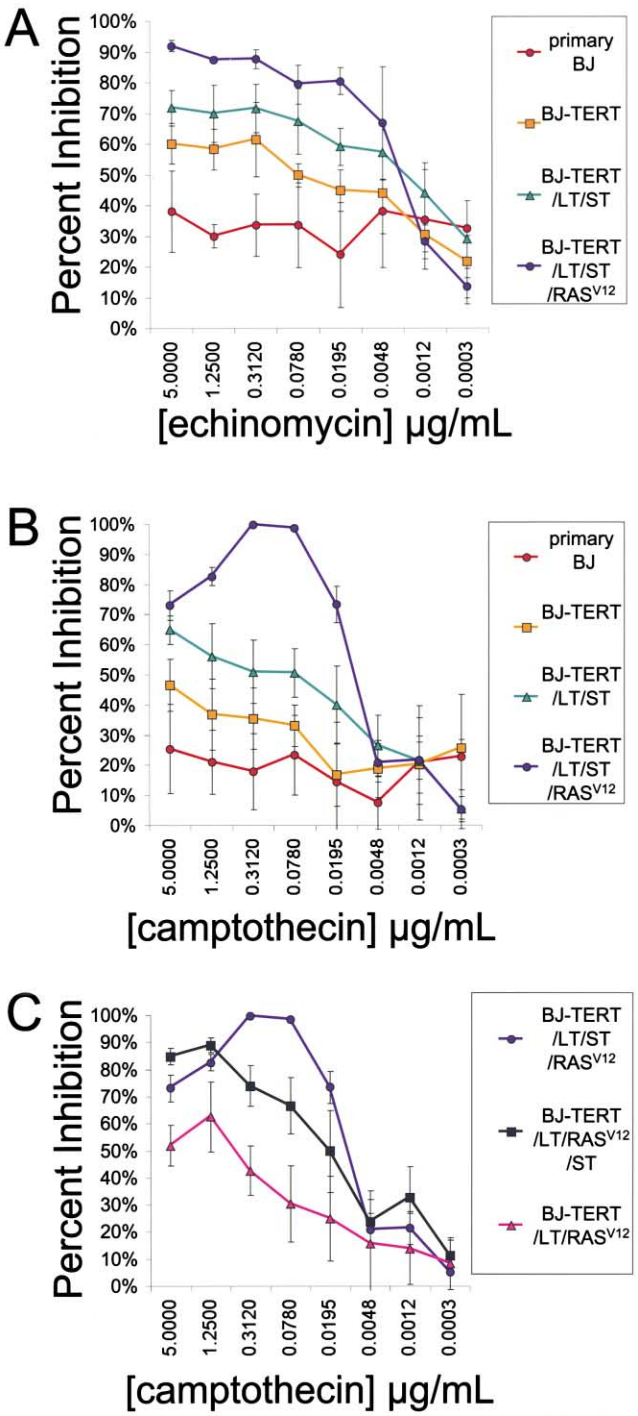

Figure 3. Effect of echinomycin and camptothecin on engineered cells

The indicated cells were treated with echinomycin (**A**) or camptothecin (**B** and **C**) in 384-well plates for 48 hr. Percent inhibition of cell viability, measured using calcein AM, is shown. Error bars indicate one standard deviation.

A: Echinomycin-treated BJ, BJ-TERT, BJ-TERT/LT/ST, and BJ-TERT/LT/ST/RAS^{V12} cells.

B: Camptothecin-treated BJ, BJ-TERT, BJ-TERT/LT/ST, and BJ-TERT/LT/ST/RAS^{V12} cells.

C: Camptothecin-treated BJ-TERT/LT/RAS^{V12}, BJ-TERT/LT/RAS^{V12}/ST and BJ-TERT/LT/ST/RAS^{V12} cells.

of ROS in these engineered cells in the presence of these three compounds (data not shown). We discovered that these compounds become more potent (i.e., active at a lower concentration) when *hTERT* is introduced and again when RB is inactivated by introduction of LT or HPV E7. In our cells, E7 was introduced after E6, so it is possible that the increased potency of these compounds in cells harboring E7 also relies on the presence of E6, even though E6 by itself does not confer increased potency to these compounds. We determined that introduction of *hTERT* and inactivation of RB caused an increase in topoisomerase IIα expression (Figure 5A) and only a very modest increase in topoisomerase IIβ expression (data not shown). Introduction of oncogenic RAS causes a further increase in topoisomerase IIα expression, although we did not observe a further sensitization to the topoisomerase II poisons in the presence of oncogenic RAS (Figure 5A).

The compounds in group 3 are camptothecin (CPT) and a novel compound from a combinatorial library, which we have named erastin, for *e*radicator of *RA*S and *ST*-expressing cells (Figure 2). Efficient CPT-induced and erastin-induced cell death requires the presence of both ST and RAS^{V12} (Figures 3 and 4 and Table 1). Although CPT and erastin have a similar genetic basis of selectivity, they have distinct mechanisms of action. CPT is partially active in cells lacking RB function (via expression of E7), whereas erastin is not, and CPT requires 2 days to cause death in BJ-TERT/LT/ST/RAS^{V12} cells, while erastin is 100% effective within 18 hr (data not shown and Figures 3 and 4). The phosphatase inhibitor okadaic acid was capable of sensitizing otherwise resistant BJ primary cells to CPT (Figure 5E), possibly because okadaic acid upregulates TOP1 (Figure 5F). Okadaic acid does not render BJ or BJ-TERT cells sensitive to erastin (data not shown), consistent with a model in which CPT and erastin act via distinct mechanisms. Moreover, we found that the lethal compound podophyllotoxin, a tubulin inhibitor, does not sensitize BJ or BJ-TERT cells to CPT, confirming that the sensitization of BJ cells to CPT by okadaic acid is specific (data not shown) and not the result of two weak cell death stimuli having an additive, but functionally irrelevant, effect.

In attempting to understand the molecular basis for the increased sensitivity to CPT of RAS^{V12}- and ST-expressing cells, we determined the expression level in our engineered cells of topoisomerase I (TOP1), the putative target of CPT (Andoh et al., 1987; Bjornsti et al., 1989; Champoux, 2000; D'Arpa et al., 1990; Eng et al., 1988; Hsiang et al., 1989; Hsiang and Liu, 1988; Liu et al., 2000; Madden and Champoux, 1992; Tsao et al., 1993). We discovered that cells expressing both RAS^{V12} and ST upregulate TOP1 (Figure 5B). As CPT's putative mechanism of action in other cell types involves a gain of function, namely introduction of double-strand DNA breaks in a TOP1-dependent manner (Liu et al., 2000), upregulation of TOP1 could explain the increased sensitivity of RAS^{V12}- and ST-expressing cells to CPT. In support of this interpretation, we found that genetic inactivation of TOP1 with a small interfering RNA (siRNA) in BJ-TERT/LT/ST/RAS^{V12} cells confers partial resistance to CPT (Figures 5C and 5D).

We sought to characterize the type of cell death induced by CPT and erastin in tumorigenic BJ-TERT/LT/ST/RAS^{V12} cells (Figure 6). In other contexts, CPT has been found to induce apoptotic cell death (Traganos et al., 1996), which is characterized by alterations in nuclear morphology including pyknosis, karyorhexis, and/or margination of chromatin (Majno and Joris,

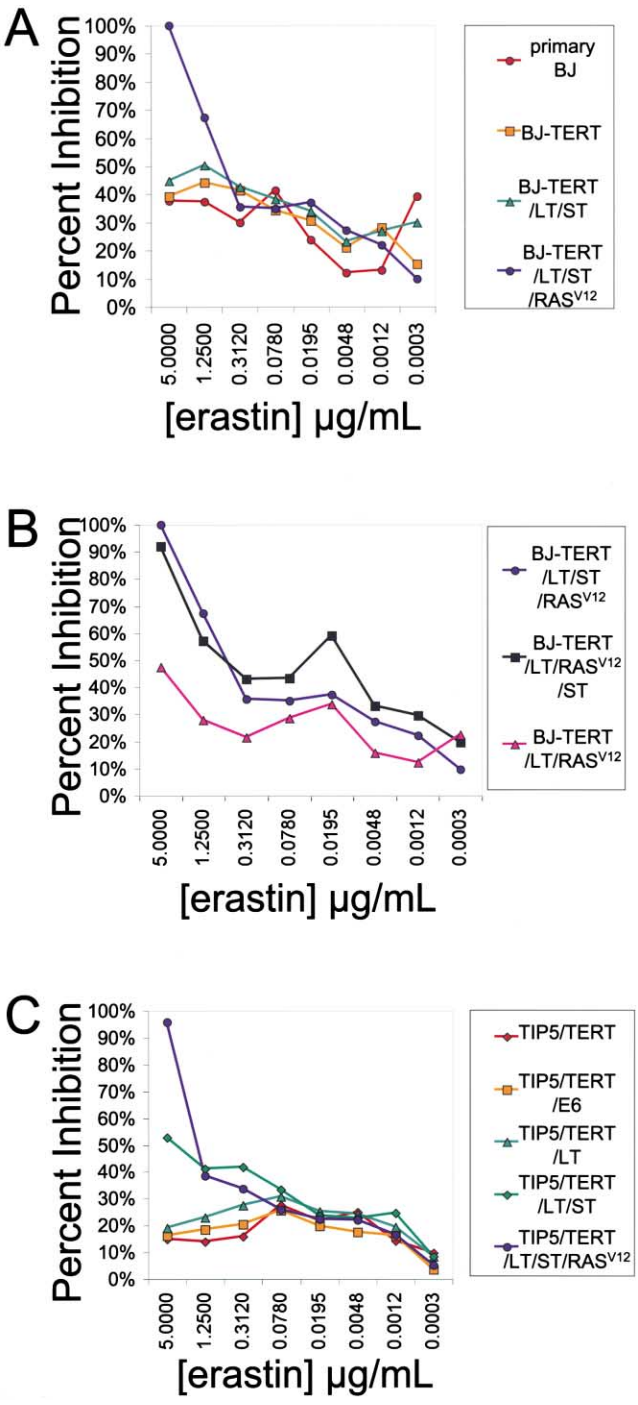

Figure 4. Effect of erastin on engineered cells

The indicated cells were treated with erastin in 384-well plates for 48 hr. Percent inhibition of cell viability, measured using calcein AM, is shown. Error bars indicate one standard deviation.

A: Erastin-treated BJ, BJ-TERT, BJ-TERT/LT/ST, and BJ-TERT/LT/ST/RAS^{V12} cells.

B: Erastin-treated BJ-TERT/LT/RAS^{V12} cells (lacking ST), BJ-TERT/LT/RAS^{V12}/ST (tumorigenic cells), and BJ-TERT/LT/ST/RAS^{V12} (tumorigenic cells).

C: Independently derived TIP5/TERT, TIP5/TERT/E6, TIP5/TERT/LT, TIP5/TERT/LT/ST, and TIP5/TERT/LT/ST/RAS^{V12} cells.

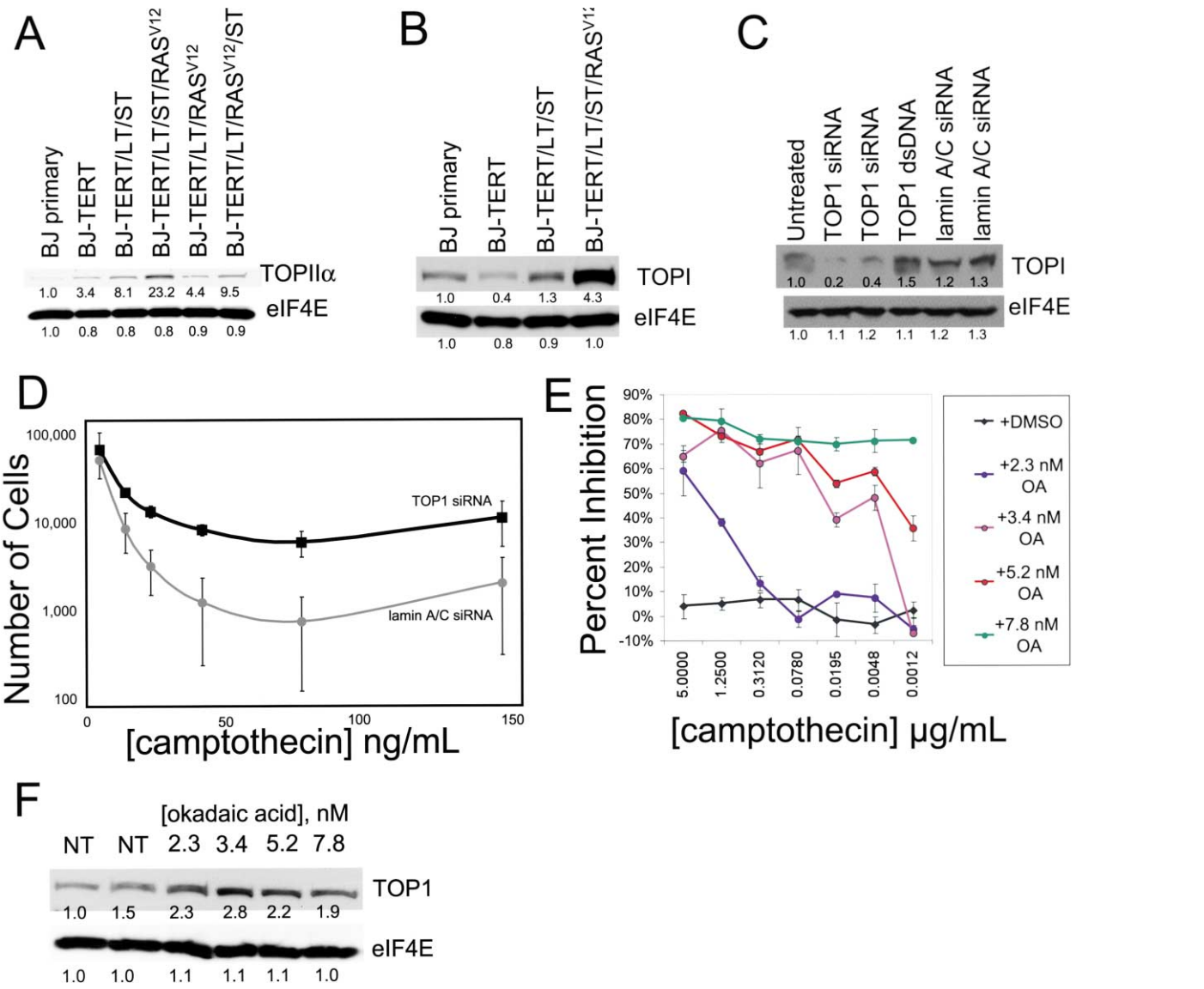

Figure 5. Protein targets of tumor-selective compounds are upregulated in engineered tumorigenic cells

A–C: Western blot of lysates from BJ, BJ-TERT, BJ-TERT/LT/ST, BJ-TERT/LT/ST/RAS^{V12}, BJ-TERT/LT/RAS^{V12}, and BJ-TERT/LT/RAS^{V12}/ST cells with an antibody directed against topoisomerase IIα (**A**) or TOPI (**B** and **C**). In (**C**), cells were transfected with an siRNA directed against TOPI, lamic A/C, or with a control double-strand DNA duplex of the same length (TOPI dsDNA). In each case, the blot was probed with an antibody against eIF-4E to identify differences in the amount of protein loaded. The relative amount is quantitated below each band.

D: A TOPI siRNA prevents cell death caused by camptothecin in engineered tumor cells. Cell number was determined after transfection with an siRNA directed against TOPI and treatment with the indicated concentrations of camptothecin.

E: Okadaic acid, an inhibitor of PP2A and other cellular phosphatases, sensitizes primary human cells to camptothecin. BJ primary cells were treated simultaneously with the indicated concentrations of both camptothecin and okadaic acid, and the effect on calcein AM viability staining was determined. Although okadaic acid kills BJ cells at the highest concentrations tested, at 3.4 nM it has no effect on its own, but it renders BJ cells sensitive to camptothecin.

F: Okadaic acid stimulates expression of TOP1. BJ primary cells were treated with the indicated concentrations of okadaic acid, and the expression level of TOPI was determined by Western blot. The relative amount is quantitated below each band.

1995). To determine whether erastin or CPT induces apoptosis in our system, we monitored the nuclear morphology of CPT- and erastin-treated tumorigenic cells using fluorescence microscopy. Although karyorhexis and margination of chromatin were clearly visible in CPT-treated cells, no such morphological alternation was visible in erastin-treated cells (Figure 7A). Since nuclear morphological change is required of apoptotic cells, we conclude that cell death induced by erastin is nonapoptotic. Further supporting this conclusion were our observations that CPT, but not erastin, induces DNA fragmentation (i.e., formation of a DNA ladder), that a pan-caspase inhibitor (50 μM Boc-Asp(Ome)-fluoromethyl ketone, Sigma #B2682 [Chan et al., 2001]) partially blocked cell death induced by CPT, but not by erastin (data not shown), and that CPT, but not erastin, caused an increase in Annexin V staining (Figure 7B) and the appearance of cleaved, active caspase 3 (Figure 7C).

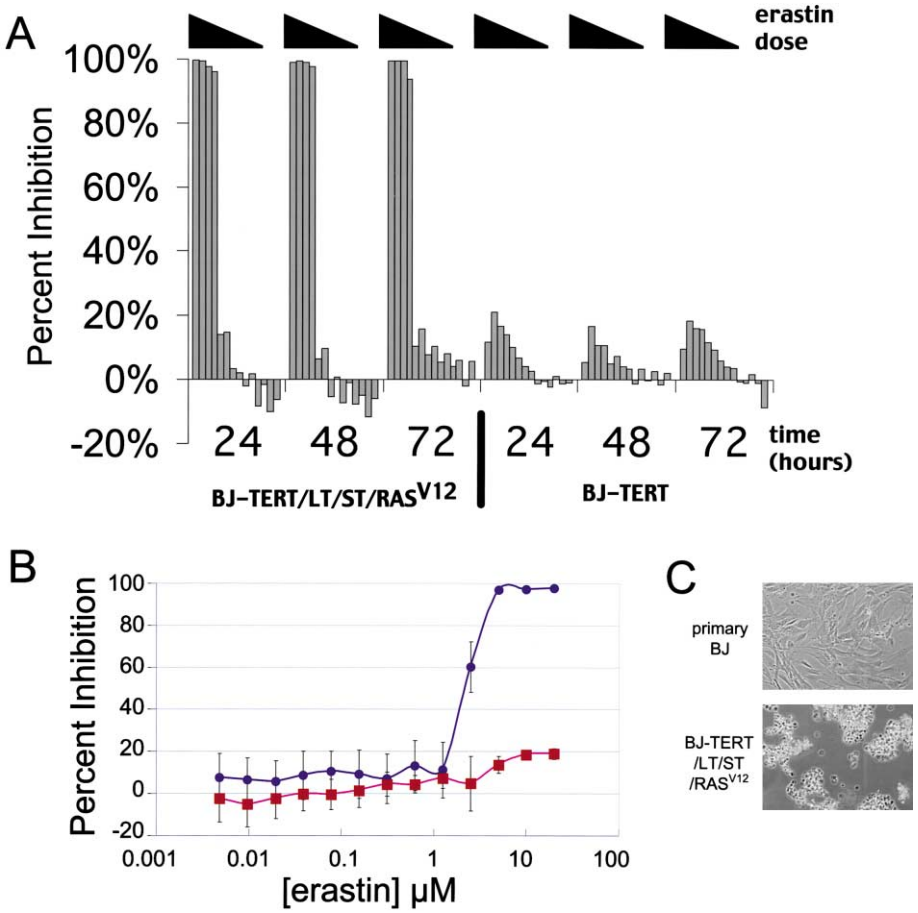

Figure 6. Erastin induces rapid cell death in a ST/RAS^{V12}-dependent fashion

A: Time-dependent effect of erastin on BJ-TERT and BJ-TERT/LT/ST/RAS^{V12} cells. Cells were seeded in 384-well plates in the presence of the indicated concentrations of erastin. Inhibition of cell viability was determined after 24, 48, and 72 hr using calcein AM.

B: Effect of erastin on Alamar Blue viability staining in BJ-TERT (red) and BJ-TERT/LT/ST/RAS^{V12} (blue) cells.

C: Photograph of BJ-TERT/LT/ST/RAS^{V12} and BJ primary cells treated with erastin. Cells were allowed to attach overnight, then treated with 9 µM erastin for 24 hr and photographed.

Erastin's ability to induce nonapoptotic cell death is selective for ST- and RAS^{V12}-expressing cells. Longer treatments and higher concentrations of erastin had little effect on the viability of cells lacking RAS^{V12} and ST, confirming the qualitative nature of erastin's selectivity (Figures 6A and 6C). As erastin-treated cells do not undergo apoptosis, we sought to confirm that erastin genuinely induces cell death, rather than cell detachment. We quantitated cell viability in the presence of erastin using Alamar Blue (Ahmed et al., 1994), a viability dye that measures intracellular reductive potential. We found that erastin exhibited selective lethality in tumorigenic BJ-TERT/LT/ST/RAS^{V12} cells relative to BJ-TERT cells in this homogeneous Alamar Blue viability assay (Figure 6B). BJ-TERT/LT/ST/RAS^{V12} cells treated with erastin for 18 hr rounded up and detached (Figure 6C), failed to exclude the vital dye Trypan Blue, displayed a loss of mitochondrial membrane potential as assayed by the potentiometric dye JC-1, and had a small cell size characteristic of dead cells (data not shown). We determined that the loss of viability induced by erastin is irreversible once completed, in that BJ-TERT/LT/ST/RAS^{V12} cells treated with erastin for 24 hr rounded up, detached, and were unable to recover when replated in erastin-free medium. Thus, erastin induces rapid (12–24 hr), irreversible, nonapoptotic cell death in a ST- and RAS^{V12}-dependent fashion.

Discussion

We have demonstrated that it is possible to identify compounds with increased potency and activity in the presence of specific genetic elements. Although previous reports indicated that it may be possible to identify such genotype-selective compounds in the case of one genetic element of interest (Simons et al., 2001; Stockwell et al., 1999; Torrance et al., 2001), our efforts here provide a systematic testing of synthetic lethality using more than 23,000 compounds and six cancer-related genetic elements.

The nine selective compounds we identified help to define consequences of introducing TERT, LT, ST, E6, E7, and oncogenic RAS into normal human cells. One effect of these genetic changes is to increase the rate of cell proliferation and to allow sensitivity to small molecules that inhibit DNA synthesis. Although it is well established that such agents preferentially target rapidly replicating tumor cells, it is reassuring to see this principle emerge from this unbiased screening approach. Moreover, we are able to readily discard these less interesting compounds because they lack a clear genetic basis of selectivity.

Second, we discovered that expression of hTERT and either E7 or LT sensitizes cells to topoisomerase II poisons. Since loss or inactivation of RB (Sellers and Kaelin, 1997; Sherr, 2001) and activation of telomerase (Hahn and Weinberg, 2002; Harley, 1994) are found in most human cancers, these observations may explain, in part, the activity of these agents in a diverse range of human tumor types.

Third, we discovered that camptothecin is selectively lethal to cells harboring both *ST* and oncogenic *RAS* because of the combined effect of these two genes on expression of topoisomerase I. Rapidly dividing tumor cells use topoisomerase I to

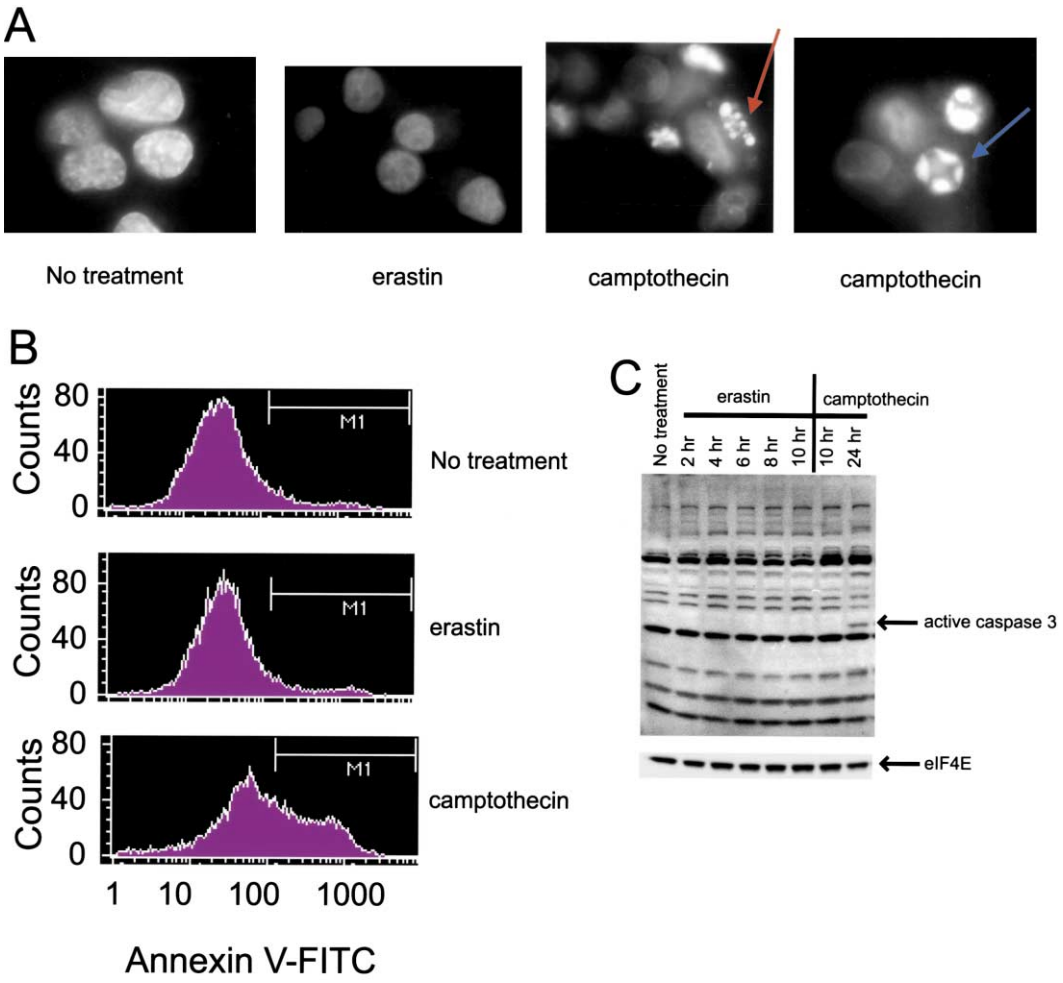

Figure 7. Camptothecin, but not erastin, induces characteristics of apoptosis

A: Camptothecin-treated, but not erastin-treated, BJ-TERT/LT/ST/RAS^{V12} cells displayed fragmented nuclei (10%–20% of total nuclei, red and blue arrows) as shown.

B: Camptothecin-treated, but not erastin-treated, BJ-TERT/LT/ST/RAS^{V12} cells display Annexin V staining. The percentage of cells in the indicated M1 region were 6%, 6%, and 38% in untreated, erastin-treated (9 μM), and camptothecin-treated (1 μM), respectively.

C: Camptothecin-treated, but not erastin-treated, BJ-TERT/LT/ST/RAS^{V12} cells harbor activated caspase 3. Lysates of camptothecin- and erastin-treated samples were analyzed by Western blot with an antibody directed against the active, cleaved form of caspase 3. The blot was reprobed with an antibody directed against eIF4E to control for loading levels.

unwind supercoiled DNA to effect continuous and rapid cell division. When these two pathways are simultaneously altered, topoisomerase I is upregulated, perhaps indirectly, and such tumor cells are rendered sensitive to topoisomerase I poisons.

These observations suggest that one aspect of the ability of ST to transform human cells along with RAS^{V12}, LT, and hTERT may be the effect of ST and RAS^{V12} on expression of topoisomerase I. Mutations in *HRAS* and *KRAS* have been described in many types of human cancers. Moreover, the inactivation of *PPP2R1B*, a component of PP2A, has recently been reported in colon and lung tumors (Wang et al., 1998), while mutations in a different PP2A subunit have been described in melanoma, lung, breast, and colon cancers (Calin et al., 2000; Kohno et al., 1999; Ruediger et al., 2001a, 2001b). At present, it remains unclear whether simultaneous alteration of these two pathways occurs at high frequency in human tumors or whether cancers in which both of these pathways are perturbed show increased susceptibility to these compounds.

Finally, we have identified a novel compound, which we named erastin, that is only lethal to cells expressing both ST and RAS^{V12}. Treatment of cells with this compound failed to kill cells lacking RAS^{V12} and ST, even when used at concentrations 8-fold higher than was required to observe an effect on cells expressing both RAS^{V12} and ST, indicating a degree of specificity. The lethal effect of erastin is rapid and irreversible once obtained.

For both CPT and erastin, we identified synergy between pathways altered by expression of RAS^{V12} and ST. Expression of RAS^{V12} leads to the activation of several well-characterized signaling pathways, including the RAF-MEK-MAPK signaling cascade, the phosphatidylinositol 3-kinase (PI3K) signaling pathway, and the Ral-guanine dissociation factor pathway (Ral-GDS). Each of these pathways have been implicated in human

cancers, and recent work demonstrates that these pathways work in concert in this system of cell transformation (Hamad et al., 2002). In addition, ST binds to and inactivates PP2A, a widely expressed serine-threonine phosphatase. Although the specific enzymatic targets of PP2A that are perturbed upon expression of ST are not yet known, there is substantial overlap among pathways altered by PP2A and RAS (Millward et al., 1999). Understanding further the mechanism by which erastin induces death in cells harboring alterations of these two signaling pathways may provide clues to the nature and extent of functional overlap between these two pathways.

Finally, these studies have provided a link between ST and RAS^{V12} signaling and a rapid and selective, nonapoptotic cell death pathway operative in human fibroblasts. Identifying novel mechanisms for killing tumor cells, particularly in a genotype-selective fashion, would be of value for understanding tumor cell biology and development of new classes of antitumor agents. Some have argued that most existing antitumor agents kill tumor cells via apoptosis (Makin, 2002), highlighting the potential importance of our finding that erastin acts through a novel, nonapoptotic pathway. The discovery of these signaling interactions was made possible by our combined use of chemical genetic and molecular genetic approaches to tumor cell biology. Although we have made use of *hTERT*, *LT*, *ST*, *E6*, *E7*, and RAS^{V12} as transforming genes, future studies can make use of a wide variety of cancer-associated alleles using this methodology in order to define the signaling networks that emanate from many oncogenes and tumor suppressors. Such studies may ultimately unravel details of these and other critical signaling networks altered by oncogenic mutations.

Experimental procedures

Constructs and retroviruses

Expression constructs for hTERT, LT, ST, SV40 Early Region, and $HRAS^{V12}$ were used as previously described (Hahn et al., 1999, 2002). hTERT-pWZL-Blastϵ, E6-pWZL-zeoϵ, and E6E7-pWZL-Zeoϵ were previously described (Lessnick et al., 2002). The E6 and LT cDNAs were cloned into the pWZL-Hygroϵ retroviral vector (a kind gift from J. Morgenstern, Millenium Pharmaceuticals). Vesicular stomatitis virus-G glycoprotein pseudotyped retroviruses were prepared, and infections carried out as described previously (Lessnick et al., 2002).

Cell lines

TIP5 primary fibroblasts (Lessnick et al., 2002) were prepared from discarded neonatal foreskins and were immortalized by infection with hTERT-pWZL-blastϵ or hTERT-pBabe-hygro retroviruses and selection with either blasticidin or hygromycin, respectively. BJ cells were a gift of Jim Smith. hTERT-immortalized fibroblasts were infected with the indicated retroviruses and selected for the appropriate markers. All BJ derivatives were cultured in a 1:1 mixture of DMEM and M199 supplemented with 15% inactivated fetal bovine serum, penicillin, and streptomycin (pen/strep). TIP5 cells were grown in DMEM containing 10% FBS and pen/strep. All cell cultures were incubated at 37°C in a humidified incubator containing 5% CO_2.

Compound libraries

An annotated compound library (ACL) comprising 1540 compounds, an NCI diversity set of 1990 compounds obtained from the National Cancer Institute, and a combinatorial library (Comgenex International, Inc.) containing 20,000 compounds were used in the tumor-selective synthetic lethal screens. All compound libraries were prepared as 4 mg/ml solutions in DMSO in 384-well polypropylene plates (columns 3–22) and stored at −20°C. Camptothecin (cat# C9911, MW 348.4), doxorubicin (cat# D1515 MW 580.0), daunorubicin (cat# D8809, MW 564.0), mitoxantrone (cat# M6545, MW 517.4), okadaic acid (cat# O4511, MW 805.0), echinomycin (cat# E4392, MW 1101), and sangivamycin (cat# S5895, MW 309.3) were obtained from Sigma-Aldrich Co. Bouvardin (MW 772.84) and NSC146109 (MW 280.39) were obtained from the National Cancer Institute's Developmental Therapeutics Program. Erastin (MW 545.07) was obtained from Comgenex International, Inc.

Calcein AM viability assay

Calcein acetoxylmethyl ester (AM) is a cell membrane-permeable, nonfluorescent compound that is cleaved by intracellular esterases to form the anionic, cell-impermeable, fluorescent compound calcein. Viable cells are stained by calcein because of the presence of intracellular esterases and because the intact plasma membrane prevents fluorescent calcein from leaking out of cells (Wang et al., 1993). Cells were seeded in 384-well plates using a Zymark Sciclone ALH, treated with each compound in triplicate at 4 μg/ml in the primary screen for 2 days, washed with phosphate-buffered saline on a Packard Minitrak with a 384-well washer, and incubated for 4 hr with 0.7 μg/ml calcein (Molecular Probes). Total fluorescence intensity in each well was recorded on a Packard Fusion platereader and converted to a percent inhibition of signal by subtracting the instrument background and dividing by the average signal obtained when cells were not treated with any compound.

Alamar Blue viability assay

Alamar Blue is reduced by mitochondrial enzyme activity in viable cells, causing both colorimetric and fluorescent changes (Nociari et al., 1998). Cells were seeded at a density of 6000 cells (50 μl) per well in a 384-well black, clear-bottom plate using a syringe bulk dispensor (Zymark). Ten microliters were removed from a 2-fold serially diluted erastin plate (6× final concentration) using a 384 fixed cannula head, making the final concentration 20 μg/ml in the well with highest concentration. The plates were incubated for 24 hr. Alamar Blue (Biosource International) was added to each well by diluting 1:10 and incubated for 16 hr at 37°C. Fluorescence intensity was determined using a Packard Fusion platereader with an excitation filter centered on 535 nm and an emission filter centered on 590 nm. Average percentage inhibition at each concentration was calculated. Error bars indicate one standard deviation. The Alamar Blue assay does not involve washing the cells.

Screening

Replica daughter plates were prepared with a Zymark Sciclone ALH and integrated Twister II by diluting stock plates 50-fold in medium lacking serum and pen/strep to obtain a compound concentration in daughter plates of 80 μg/ml with 2% DMSO. Assay plates were prepared by seeding cells in black, clear bottom 384-well plates in columns 1–23 (6000 cells/well in 57 μl) using a syringe bulk dispenser. Columns 3–22 were treated with compounds from a daughter library plate by transferring 3 μl from the daughter library plate using 384-position fixed cannula array. The final compound concentrations in assay plates were thus 4 μg/ml. The assay plates were incubated for 48 hr at 37°C in humidified incubator containing 5% CO_2. Plate processing for the calcein AM viability assay was performed using an integrated Minitrak/Sidetrak robotic system from Packard Bioscience (Perkin Elmer). Assay plates were washed with phosphate-buffered saline, and 20 μl of calcein AM (0.7 μg/ml) per well was added. Plates were incubated at room temperature for 4 hr. Fluorescence intensity was determined using a Fusion platereader with filters centered on an excitation of 485 nm and an emission of 535 nm.

Retesting of compounds in a dilution series

Compounds to be retested were purchased from manufacturers. Stocks were prepared in DMSO at a concentration of 1 mg/ml in 384-well polypropylene plates with a 16-point, 2-fold dilution dose curve of each compound in a column, in duplicates. Columns 1, 2, 23, and 24 were left empty for controls. Daughter retest plates were prepared from stock retest plate by diluting 66.6-fold in DMEM in 384-well deep-deep well plates (4.5 μl transfer into 300 μl). Cells were seeded at a density of 6000 per well in 40 μl, and 20 μl was added from a daughter retest plate. The plates were incubated for 2 days at 37°C with 5% CO_2.

Data analysis

Mean RFU (relative fluorescence units) for untreated cells was calculated by averaging columns 1, 2, and 23 (wells with cells but lacking compounds).

The calcein background was calculated by averaging column 24 (wells with calcein, but lacking cells). Percentage inhibition of each well was calculated as (1 − [RFU − calcein control]/[untreated cell − calcein control]·100). Compounds causing at least 50% inhibition of calcein staining in the primary screen were tested for selectivity toward BJ-TERT/LT/ST/RAS^{V12} engineered tumor cells by testing in BJ primary and BJ-TERT/LT/ST/RAS^{V12} cells at a range of concentrations. Selective compounds were retested in all engineered cell lines.

Nuclear morphology assay

200,000 tumorigenic BJ-TERT/LT/ST/RAS^{V12} cells were seeded in 2 ml on glass coverslips in each well of a 6-well dish, treated with nothing (NT), 9 μM erastin, or 1.1 μM camptothecin (CPT) in growth medium for 18 hr while incubating at 37°C with 5% CO_2. Nuclei were stained with 25 μg/ml Hoechst 33342 (Molecular Probes) and viewed using an oil immersion 100× objective on a fluorescence microscope.

Cell size measurements

200,000 BJ-TERT/LT/ST/RAS^{V12} cells were seeded in 6-well dishes in 2 ml growth medium only (No treatment), with 9 μM erastin, or with 1.1 μM camptothecin (CPT). After 24 hr, cells were released with trypsin/EDTA and diluted to 10 ml in growth medium, and the cell size distribution of each sample was determined on a Coulter Counter.

Cell counting assay for camptothecin activity

BJ-TERT/LT/ST/RAS^{V12} cells were seeded in 6-well dishes (200,000 cells/well; 2 ml per well) and transfected in serum- and antibiotic-free medium using Oligofectamine (Life Technologies), with 100 nM siRNA per well in a total volume of 1 ml. 500 μl of medium containing 30% FBS was added 4 hr after transfection. Cells were treated with the indicated concentrations of camptothecin 30 hr after transfection. 500 μl of a 5× solution of the desired camptothecin concentration was added to each well. Cells were removed with trypsin-EDTA and counted using a hemacytometer 75 hr after transfection. Control experiments indicated the transfection efficiency was approximately 10%.

Western blot analysis

Caspase-3

BJ-TERT/LT/ST/RAS^{V12} cells were seeded prior to the experiment at 5×10^5 cells in 60 mm dishes. The cells were treated with 5 μg/ml erastin (9 μM) for 2, 4, 6, 8, or 10 hr. One dish was maintained for camptothecin treatment (0.4 μg/ml for 24 hr) as a positive control. Cells were lysed after each time point in lysis buffer (50 mM HEPES KOH [pH 7.4], 40 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 1.5 mM Na_3VO_4, 50 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate, and protease inhibitor tablet [Roche]). Protein content was quantified using a Biorad protein assay reagent. Equal amounts of protein were resolved on 16% SDS-polyacrylamide gel. The electrophoresed proteins were transblotted onto a PVDF membrane, blocked with 5% milk, and incubated with anti-active caspase-3 polyclonal antibody (BD Pharmingen) at 1:1500 dilution overnight at 4°C. The membrane was then incubated in anti-rabbit-HRP (Santa Cruz Biotechnology) at 1:3000 dilution for 1 hr and developed with an enhanced chemiluminescence mixture (NEN life science, Renaissance). To test for equivalent loading in each lane, blots were stripped, blocked, and probed with an anti-eIF-4E antibody (BD Transduction laboratories) at 1:1000 dilution.

Topoisomerase-IIα

BJ, BJ-TERT, BJ-TERT/LT/ST, BJ-TERT/LT/ST/RAS^{V12}, BJ-TERT/LT/RAS^{V12}, and BJ-TERT/LT/RAS^{V12}/ST cells were seeded at 1×10^6 cells per dish in 60 mm dishes. After overnight incubation of the cells at 37°C with 5% CO_2, the cells were lysed as described above and proteins resolved on a 10% polyacrylamide gel. The membrane was incubated with monoclonal anti-human topoisomerase IIα p170 antibody (TopoGEN) at 1:1000 dilution overnight at 4°C and then with anti-mouse HRP (Santa Cruz Biotechnology).

Topoisomerase 1 (TOP1)

A 21-nucleotide double-stranded siRNA directed against TOP1 (nucleotides 2233–2255, numbering from the start codon, GenBank accession J03250) was synthesized (Dharmacon, purified and desalted/deprotected) and transfected (100 nM) into BJ-TERT/LT/ST/RAS^{V12} cells in six-well dishes with oligofectamine (Life Technologies). After 75 hr, cells were lysed and the expression level of TOP1 determined by Western blot (Topogen, Cat# 2012-2, 1:1000 dilution). The protein loading level was determined by stripping and reprobing the same blot with an antibody directed against eIF-4E (BD Biosciences, Cat# 610270, 1:500 dilution). Alternatively, 1×10^6 cells were seeded in 60 mm dishes and grown overnight at 37°C with 5% CO_2, then lysed with 150 μl of lysis buffer. Cells were removed with a scraper and transferred to microcentrifuge tubes and incubated on ice for 30 min. The protein contents in the lysates were quantified using a Biorad protein estimation assay reagent. Equal amounts of protein were loaded on 10% gradient SDS-polyacrylamide gel. The electrophoresed proteins were transblotted onto PVDF membrane. After blocking with 5% dry milk, the membrane was incubated with mouse anti-Human topoisomerase I antibody (Pharmingen) overnight at 4°C, then with anti-mouse peroxidase conjugate antibody (Santa Cruz Biotechnology).

Annexin V-FITC Apoptosis Assay

BJ-TERT/LT/ST/RAS^{V12} cells were seeded at 1×10^6 cells per dish in 100 mm dishes and allowed to grow overnight. Cells were treated with erastin (5 or 10 μg/ml) for 6, 8, or 11 hr. A camptothecin-treated (0.4 μg/ml) control was maintained, treated at the time of seeding for 20 hr. After the treatment, cells were harvested with trypsin/EDTA and washed once with fresh medium containing serum and then twice with phosphate-buffered saline. Cells were resuspended in 1× binding buffer (BD Pharmingen) at a concentration of 1×10^6 cells/ml. 100 μl (1×10^5 cells) was incubated with 5 μl of Annexin V-FITC (BD Pharmingen) and propidium iodiode (BD Pharmingen) for 15 min in the dark at room temperature. Then 400 μl of the 1× binding buffer was added and the cells analyzed by flow cytometry (Becton-Dickinson). Data were acquired and analyzed using Cellquest software. Only viable cells that did not stain with propidium iodiode were analzyed for Annexin V-FITC staining using the FL1 channel.

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