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RNAi Screening Identifies TAK1 as a Potential Target for the Enhanced Efficacy of Topoisomerase Inhibitors

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Abstract

In an effort to develop strategies that improve the efficacy of existing anticancer agents, we have conducted a siRNA-based RNAi screen to identify genes that, when targeted by siRNA, improve the activity of the topoisomerase I (Top1) poison camptothecin (CPT). Screening was conducted using a set of siRNAs corresponding to over 400 apoptosis-related genes in MDA-MB-231 breast cancer cells. During the course of these studies, we identified the silencing of *MAP3K7* as a significant enhancer of CPT activity. Follow-up analysis of caspase activity and caspase-dependent phosphorylation of histone H2AX demonstrated that the silencing of *MAP3K7* enhanced CPT-associated apoptosis. Silencing *MAP3K7* also sensitized cells to additional compounds, including CPT clinical analogs. This activity was not restricted to MDA-MB-231 cells, as the silencing of *MAP3K7* also sensitized the breast cancer cell line MDA-MB-468 and HCT-116 colon cancer cells. However, *MAP3K7* silencing did not affect compound activity in the comparatively normal mammary epithelial cell line MCF10A, as well as some additional tumorigenic lines. *MAP3K7* encodes the TAK1 kinase, an enzyme that is central to the regulation of many processes associated with the growth of cancer cells (*e.g.* NF- κ B, JNK, and p38 signaling). An analysis of TAK1 signaling pathway members revealed that the silencing of *TAB2* also sensitizes MDA-MB-231 and HCT-116 cells towards CPT. These findings may offer avenues towards lowering the effective doses of Top1 inhibitors in cancer cells and, in doing so, broaden their application.

Keywords

RNAi; siRNA; Screen; Camptothecin; TAK1; MAP3K7; TAB2; TRAF6

INTRODUCTION

For more than half a century, cancer chemotherapy has focused on the use of agents that target essential biomolecules present both in cancer and normal cells. Accordingly, these

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers Web site along with the published article.

treatments are limited by deleterious side effects. An improved understanding of cancer biology has begun to circumvent these limitations through cancer-specific molecularly targeted therapies [1]. Examples of rationally designed agents include BCR-ABL, EGFR (ErbB1), and HER-2 (ErbB2) inhibitors, which have been approved for the treatment of chronic myelogenous leukemia, lung cancer, and breast cancer, respectively (reviewed in [2, 3]). However, despite the promise of these efforts, the scope of discriminating therapeutics remains limited and broadly cytotoxic agents remain at the center of the present therapies.

Synthetic siRNAs induce the sequence-specific cleavage of target mRNA transcripts to generate gene-specific loss of function (reviewed in [4]). Recently, siRNA-based RNAi screening has been used to find molecular targets that improve the efficacy of both targeted and cytotoxic agents [5–19]. For example, RNAi screening revealed targets that sensitize triple negative breast cancer cells to camptothecin (CPT) [15] and lung cancer cells to paclitaxel [13]. In an analogous study, the inhibition of ceramide transport significantly enhanced paclitaxel activity in colon, breast, and lung cancer cell lines [12]. Conversely, this study also discovered a number of paclitaxel antagonists related to spindle checkpoint disruption. In both studies, small molecule inhibitors of the identified targets reproduced siRNA-mediated activity, clearly demonstrating the value of RNAi screening in molecular target discovery.

To add to these current efforts, we have conducted an additional siRNA-based chemosensitization screen in the context of the classical cytotoxic agent CPT, a topoisomerase I (TOP1) inhibitor that induces replication-mediated DNA double-strand breaks (reviewed in [20]). Water-soluble analogs of CPT are used in the treatment of cancer. These include topotecan, used in the treatment of ovarian and lung cancers, and irinotecan, used in the treatment of colorectal cancer. Other CPT analogs, and non-CPT Top1 inhibitors, are in preclinical development [20–22]. Nonetheless, these agents suffer from significant side effects that limit their tolerable dose and efficacy. By conducting an RNAi screen in the context of CPT, we hoped to identify novel targets that improve the efficacy of camptothecins. This study identified the silencing of *MAP3K7* as a significant enhancer of CPT activity in the breast cancer cell line MDA-MB-231. Follow-up analysis demonstrated similar activity in the colon cancer cell line HCT-116, but not in all cell lines tested. *MAP3K7* encodes the TAK1 MAP3K, which is a key regulator of important cellular pathways including NF- κ B, JNK, and p38. An investigation of other members of the TAK1 signaling pathway revealed that the silencing of *TAB2* also sensitizes MDA-MB-231 and HCT-116 cells to CPT. Such targets may offer strategies towards lowering the effective doses of camptothecins and, in doing so, broaden their application.

MATERIALS AND METHODS

Cell Lines and Reagents

MDA-MB-231 breast cancer cells and HCT-116 colon cancer cells were obtained from the NCI Developmental Therapeutics Program (DTP) (<http://dtp.nci.nih.gov/>) and were maintained in RPMI1640 containing 5% fetal bovine serum (FBS) (both from Invitrogen, Carlsbad, CA). An additional line of MDA-MB-231 cells, which were maintained independently for several years and used for phenotype confirmation, was obtained from the laboratory of Dr. J. Weinstein (CCR, NCI). The normal mammary epithelial MCF10A cell line was obtained from Dr. P. Steeg (CCR, NCI) and grown as described [23]. MDA-MB-468 cells were obtained from Dr. S. Lipkowitz (CCR, NCI) and grown in RPMI containing 10% FBS. TAK1 and α -tubulin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). phospho-JNK and phospho-p38 antibodies were obtained from Cell Signaling Technology (Beverly, MA). γ -H2AX antibody was obtained from Abcam (Cambridge, MA). Z-VAD-FMK was obtained from BD Biosciences (San Jose,

CA). All siRNAs were obtained from Qiagen Inc. (Germantown, MD). The sequences of siRNAs used in this study are detailed in Supplemental Table S1 and Supplemental Table S2.

siRNA Screening

The CPT chemosensitization screen was performed using the Human Apoptosis Set Library (Qiagen Inc., Germantown, MD; see Supplemental Table S1 for genes targeted). Screening was conducted in 96 well plates. Transfections were performed by pre-complexing siRNA (2 pmol) with 0.7 μ L oligofectamine lipid transfection reagent (Invitrogen) in 50 μ L of serum free RPMI in individual plate wells for 30 min at ambient temperature. Next, MDA-MB-231 cells (4,500) were added in 50 μ L RPMI supplemented with 10% FBS to yield transfection mixtures consisting of 20 nM siRNA in RPMI with 5% FBS. This final mixture was incubated at ambient temperature for 45 min before being placed at 37 °C in a humidified atmosphere containing 5% CO₂. Two copies of the library were screened. After 48 h, the media was removed and one copy of the library received 100 μ L of fresh media containing either 200 nM CPT ($\approx$ EC₅₀, 0.1% DMSO), while vehicle only (0.1% DMSO) was added to the second copy. Cells incubated for an additional 48 h at 37 °C. After this time, cell viability was assayed (Cell Titer Blue Reagent, Promega, Madison, WI). Plate median values were used for normalization excluding the negative and positive controls. Negative and positive controls were also used for qualitative evaluation of screen behavior. Negative control siRNA was arrayed in 3 wells per plate. The negative siRNA duplex consists of 5'-r(ACGUG ACACGUUCGGAGAA)dTdT and 5'-r(UUCUCCGAACG UGUCACGU)dTdT strands. A single well on each plate containing a siRNA corresponding to the Polo-like kinase 1 gene (siPLK1.6) was used as a positive control, as transfection with this siRNA leads to a significant reduction in MDA-MB-231 viability. The assessment of TAK1 signaling complex members (*TAB1*, *TAB2* and *TAB3*) was conducted using the same protocol though each siRNA was assessed in triplicate. See Supplemental Table S2 for the sequences of siRNAs used as controls or for follow up of specific genes.

Dose Response Analysis

For dose response analyses in MDA-MB-231 cells using siMAP3K7-1, siMAP3K7-3, siTAB2-1, or siTAB2-2 transfections (20 nM siRNA) were performed as described for screening, except that 2,500 cells were seeded. After 48 h, the media was removed and 100 μ L of fresh media containing compound (0.1% DMSO) was added and the cells incubated for an additional 72 h at 37 °C. Each data point was obtained in triplicate. Cells transfected with negative control siRNA were used for comparison. Cells were assayed by MTS assay (Cell Titer 96 Aqueous One Solution Reagent, Promega, Madison, WI). The same transfection procedures were used for experiments conducted in additional cell lines (except MDA-MB-468 cells) with the number of cell plated and the amount of Oligofectamine used as follows: HCT-116: 2,500 cells per well, 0.7 μ L Oligofectamine; BT20: 3,000 cells per well, 0.5 μ L Oligofectamine; Hs578T: 3,500 cells. 0.7 μ L Oligofectamine; MCF7: 2,500 cells, 0.7 μ L Oligofectamine and MCF10A: 2,500 0.7 μ L Oligofectamine. MDA-MB-468 transfections were conducted in 384 well plates using 500 cells per well and 0.07 μ L RNAiMAX (Invitrogen) per well, cell viability was measured with Cell Titer Glo (Promega, Madison, WI). Silencing data for *MAP3K7* and *TAB2* can be found in Supplemental Table S3. Dose response analyses using 5Z-7-oxozeanol (Sigma, #O9890) were also conducted. MDA-MB-231 cell and HCT116 cells were treated by CPT in the presence of different doses of 5Z-7-oxozeanol for 48 h. MTS assay was used to determine cell viability.

Functional Analysis of TAK1 Pathway Members

MDA-MB-231 cells were transfected with siRNAs corresponding to TAK1 pathway members (see Supplemental Table S2 for details of genes targeted and sequences) as

described above. After 48 h, the media was removed and 100 μ L of fresh media containing either 50 nM CPT ($\approx$ EC₂₀, 0.1% DMSO) or vehicle only (0.1% DMSO) was added. The cells were incubated for an additional 72 h at 37 °C before assaying for cell viability (Cell Titer Blue Reagent, Promega, Madison, WI). Each data point was obtained in triplicate. The cell viability of negative control siRNA transfected cells was used for normalization. For the study of TRAF6, MDA-MB-231 cells were transfected with siRNAs corresponding to TRAF6 in 384 well plates using an analogous protocol as described above with a final volume of 40 μ L. MAP3K7 and TAB2 siRNAs were transfected in parallel for comparison. Cell viability in 384 well plates was assayed with Cell TiterGlo Reagent, Promega, Madison, WI.

Caspase 3/7 Activity

MDA-MB-231 cells were transfected with either MAP3K7-3 or negative control siRNA as described above. After 48 h, the media was removed and 100 μ L of fresh media containing either CPT or vehicle only (0.1% DMSO) was added. Caspase 3/7 activity was measured using the Caspase-Glo 3/7 assay (Promega, Madison, WI) at the indicated time points.

Analysis of Phospho-JNK, Phospho-p38, and NF- κ B Activation

MDA-MB-231 transfections were conducted in 6 well plates by scaling the 96 well plate protocol 30 fold. After 48 h, the media was removed and 3 mL of fresh media containing either 10 μ M CPT ($\approx$ EC₉₅) or vehicle only (0.1% DMSO) was added. Cells were incubated for an additional 3 h at 37 °C before lysing for Western blot analysis (phospho-JNK and phospho-p38) or harvesting for Electrophoretic Mobility Shift (EMSA) analysis as a measure of NF- κ B activation [24]. For EMSA analysis, a binding buffer containing 10 mM HEPES (pH 7.9), 60 mM KCl, 0.4 mM dithiothreitol, 10% glycerol, 2 μ g BSA, 1 μ g poly dI-dC and total cell protein extract (10 μ g) were incubated on ice for 20 min. A ³²P-end-labeled double stranded oligonucleotide (0.175 pmole) containing the Igk-kB site (5'-CTCAACA GAGGGGACTTTCCGAGAGGCCAT-3') (underlined is the classical kB site) was then added to the binding mixture and incubated on ice for an additional 20 min. The products of the reaction mixture were resolved on a 4% native acrylamide gel made up with 0.25xTBE. Gels were dried and exposed to Kodak O-mat films at -70C or phosphorimage cassettes. The AP-1 site used for control EMSA reactions was obtained from Promega and labeled and analyzed as above.

Analysis of γ -H2AX

A gamma H2AX specific antibody (Abcam, Cambridge, MA) was used to assay phosphorylation of H2AX by Western blotting. MDA-MB-231 transfections were conducted in 6 well plates by scaling the 96 well plate protocol 30 fold. After 48 h, the media was removed and 3 mL of fresh media containing either 1 μ M CPT or vehicle only (0.1% DMSO) was added. Cells were incubated for an additional 24 h at 37 °C before lysing for Western blot analysis using or incubated for 2 h before replacement of the CPT-containing media with fresh media. Cells undergoing media replacement were incubated for an additional 24 h in fresh media. For Z-VAD-FMK experiments, cells were incubated with 100 μ M Z-VAD-FMK (0.1% DMSO) for 2h prior to CPT addition and maintained in Z-VAD-FMK until harvesting for Western blot.

RNAi Efficacy and RNA Analysis

For characterization of RNAi efficacy, gene-specific transcript levels were measured using a branched DNA-based assay (QuantiGene Reagent System, Panomics, Fremont, CA). In brief, cells were lysed 48 h after siRNA transfection and 30 μ L of lysate and RNA levels were assayed following manufacturers instructions. Gene-specific mRNA levels were

normalized to human cyclophilin B (PPIB) mRNA levels (probe set nts 74–432). Multi-gene analysis of RNA levels in the absence and presence of *MAP3K7/TAK1* silencing and absence and presence of CPT was conducted using a custom nCounter assay following manufactures instructions (Nanostring, Seattle, WA). In brief, cell lysates were combined with hybridization buffer and two oligonucleotide based probe sets, the reporter code set and the custom capture probe set. Samples were incubated at 65°C overnight. Post-hybridization processing was conducted using the nCounter Prep Station and samples were analyzed using nCounter analysis system. RNA levels were measured a total of 72 hours post siRNA transfection; 24 hours post CPT addition. Gene-specific mRNA levels were normalized to a coefficient calculated from the levels of human cyclophilin A and B (*PPIA* and *PPIB*), glucuronidase, beta (*GUSB*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA levels.

Statistical Analysis

Dose response analyses were performed using GraphPad Prism software version 4.0a (GraphPad Software, San Diego, CA). All other analyses were performed using Excel (Microsoft).

RESULTS AND DISCUSSION

A siRNA screen was performed to identify gene targets that affect the activity of CPT in the breast cancer cell line MDA-MB-231. Breast cancer cell lines are sensitive to CPT [25], but due in part to ABCG2-mediated drug resistance [26, 27], the application of CPT clinical derivatives for the treatment of breast cancer has been limited. A lack of therapeutic options for advanced breast cancer, in particular triple negative breast cancer [28], has led to the reconsideration of CPT derivatives, often in combination with other chemotherapeutic agents, as a treatment for breast cancer [29]. The MDA-MB-231 cell line is a triple negative breast cancer cell line that lacks expression of estrogen receptor, progesterone receptor, and over-expressed of ERBB2 (HER2) and, as such, is frequently used as a model for the identification or preclinical assessment of new treatments for triple negative breast cancer.

Screening was performed using a library of 836 siRNAs corresponding to 418 apoptosis-related genes (2 siRNAs per gene, 1 siRNA per well). siRNAs were screened in both the presence (EC₅₀) and absence of CPT. Screening conducted in the absence of CPT served to establish the basal activity of each siRNA. The complete data for both screens is presented in Supplemental Table S1. To identify siRNAs that affected CPT activity, the percent viability of each siRNA in the presence of CPT was plotted versus the percent viability of each siRNA in the absence of CPT (Fig. 1A). An analogous Z-score plot was also generated to help illustrate significant hits (Fig. 1B). In this screen, the top sensitizer corresponded to a siRNA targeting *MAP3K7* (siMAP3K7-1) as indicated in Fig. (1) (panels **A** and **B**). The ratio of percent viability of CPT treated cells to non-CPT treated cells for siMAP3K7-1 was 0.59, and though less dramatic, a ratio of 0.84 was observed for siMAP3K7-2.

Follow-up, dose response analysis indicated that the siMAP3K7-1 siRNA induced a 6-fold decrease in CPT EC₅₀ in MDA-MB-231 cells, as compared to cells transfected with negative control siRNA (Fig. 1C), and reduced *MAP3K7* mRNA levels by 65% (Supplemental Table S3). This effect was corroborated with an additional, sequence-independent siRNA targeting *MAP3K7* (siMAP3K7-3), which yielded an eight-fold decrease in CPT EC₅₀ (Fig. 1D), and induced a 78% reduction in *MAP3K7* mRNA levels (Supplemental Table S3). Similar sensitization was observed in a line of independently maintained MDA-MB-231 cells (Supplemental Fig. S1) and in a second triple negative breast cancer cell line MDA-MB-468 (Fig. 1E). Intriguingly, the silencing of *MAP3K7* did not sensitize the comparatively normal mammary epithelial cell line MCF10A (Fig. 2A).

Moreover, a decrease in RNAi efficiency did not cause this lack of activity, as transfection with siMAP3K7-3 achieved 85% silencing of *MAP3K7* in MCF10A cells (Supplemental Table S3). Similarly, no reproducible sensitization was observed in MCF7, BT20, or Hs578T breast cancer cells, despite efficient silencing of *MAP3K7* (Supplemental Table S3; Supplemental Fig. S2). However, a 4-fold sensitization was observed in HCT-116 colon cancer cells (Fig. 2A), corresponding to an 83% reduction in *MAP3K7* levels (Supplemental Table S3).

The silencing of *MAP3K7* sensitizes MDA-MB-231 cells to the clinical CPT derivatives topotecan and irinotecan, and to a lesser extent doxorubicin. For example, siMAP3K7-3 mediated a 6-fold decrease in the EC₅₀ value of the CPT clinical derivative topotecan (Fig. 2B). A three-fold sensitization was also observed for doxorubicin (Fig. 2B), and an independent screen using the same library of siRNAs, as used for CPT screening, also identified siMAP3K7-1 as a top enhancer of doxorubicin activity in MDA-MB-231 cells (data not shown). Sensitization was less pronounced for cisplatin and 5-fluorouracil (Supplemental Fig. S3). The silencing of *MAP3K7* also sensitized HCT-116 cells to topotecan and irinotecan by 6- and 3-fold, respectively (Fig. 2C). These results imply that the inhibition of *MAP3K7* may enhance the activity of camptothecins in different cancer cells.

MAP3K7 encodes TAK1 (Transforming growth factor- β Activated Kinase 1), a mitogen-activated serine/threonine protein kinase kinase kinase (MAP3K). TAK1 is important in activating critical cellular components, including NF- κ B, JNK, and p38. The use of a drug to phenocopy effects seen following RNAi can be useful if a drug mimics the functional effects of RNAi that result from a loss of protein rather than inhibition of a specific activity. The natural fungal resorcylic lactone 5Z-7-oxozeaenol, has been reported as an inhibitor of TAK1 catalytic activity [30] and so we tested whether the combination of CPT and 5Z-7-oxozeaenol was synergistic. In neither MDA-MB-231 nor HCT-116 cells did we see evidence of synergism (Supplemental Fig. S4). In this case we hypothesize that either a different function or more than one function of TAK1 may need to be inhibited before a synergistic effect of its loss of function on CPT is evident.

To investigate further how loss of TAK1 protein augments CPT toxicity we considered the fact that TAK1 is part of a signaling complex comprising TAK1, TAB1, and either TAB2 or its homolog TAB3 [reviewed in [31]]. From the known biology of TAK1, we evaluated whether the silencing of other members of its signaling complex would also sensitize MDA-MB-231 cells towards CPT. As shown in Fig. (3A), two independent siRNAs targeting TAB2 (siTAB2-1 and siTAB2-2) sensitized MDA-MB-231 cells to the EC₂₀ dose of CPT. Follow-up analysis showed that both induced about a 6-fold decrease in CPT EC₅₀ value (Fig. 3B), corresponding to a 88% and 77% reduction in *TAB2* mRNA levels, respectively. Similar sensitization was observed in a line of independently maintained MDA-MB-231 cells (Supplemental Fig. S1).

Several large-scale expression-profiling studies have been performed in different cell model systems to probe the transcriptional response of cells to CPT [32–36]. Like the functional genomics approach used here, the aim of many of these expression studies has been to identify novel determinants of drug sensitivity. Interestingly, none of these transcription-based studies conducted in a variety of cell line model systems identified *MAP3K7* and its complex members as potential modulators of CPT activity. To establish that *MAP3K7* and *TAB2* also show minimal transcription-related response in MDA-MB-231 cells, we examined the expression of these genes in the absence and presence of CPT. For comparison, the expression of the ribonucleotide reductase subunit, *RRM2*, that we have recently shown does show a transcriptional response to CPT [15]. Unlike *RRM2* expression,

which, is highly induced by the addition of CPT, *MAP3K7* and *TAB2* expression changed only minimally following addition of CPT (Supplemental Fig. S5).

TAK1 can activate NF- κ B, JNK, and p38 signaling pathways, and the activation of these pathways, especially NF- κ B, can be anti-apoptotic [37–39]. Accordingly, siRNA-mediated knockdown of TAK1 has been found to enhance TRAIL-induced apoptosis [40]. Similarly, the inhibition of TAK1 sensitizes cells towards TNF α [41], and siRNA screen also identified the silencing of TAK1 as a general promoter of apoptosis in pancreatic cancer cells [11]. We investigated whether TAK1 silencing augments CPT-induced apoptosis. As shown in Fig. (4A), siMAP3K7-3 enhanced CPT-associated caspase-3/7 activity in MDA-MB-231 cells, as compared to cells transfected with negative control, demonstrating increased apoptosis in response to CPT in TAK1 downregulated cells.

CPT-induced DNA damage can activate NF- κ B through a process dependent on interactions between NF- κ B essential modulator (NEMO) and activated ataxia telangiectasia mutated (ATM) proteins [42]. Owing to NF- κ B's well-established ability in preventing apoptosis, we hypothesized that *MAP3K7* silencing might inhibit CPT-induced NF- κ B activation. However, as shown in Fig. (4B), no inhibition of CPT-induced NF- κ B activation was observed by electrophoretic mobility shift assay (EMSA), despite near complete elimination of detectable TAK1 protein levels by siMAP3K7-3. Moreover, the NF- κ B (IKK β) inhibitor parthenolide did not sensitize MDA-MB-231 cells towards CPT (Supplemental Fig. S6); nor, as shown in Fig. (4C), did a series of siRNAs directed against IKK β (the direct target of TAK1 associated with NF- κ B activation).

We next examined whether disrupting JNK and p38 activation could sensitize MDA-MB-231 cells to CPT. TAK1 can activate JNK and p38 by phosphorylating MAP2K3/7 and MAP2K4/6, respectively. As shown in Fig. (4C), siRNAs directed against these upstream activators of JNK and p38 had no effect on CPT activity. Nor did chemical inhibitors of JNK and p38 (Supplemental Fig. S6). In addition to these RNAi- and inhibitor-based experiments, *MAP3K7* silencing had no detectable effect on the CPT-associated phosphorylation status of either protein (Supplemental Fig. S7).

Since silencing *MAP3K7* did not detectably affect the CPT-associated activity of the known downstream, apoptosis-related components, we next examined whether *MAP3K7* down-regulation was augmenting CPT-induced DNA damage. Camptothecins generate replication-associated DNA double-strand breaks through collisions between replication forks and drug-stabilized topoisomerase I cleavage complexes [43, 44]. These breaks result in the phosphorylation of histone H2AX, yielding γ -H2AX [45, 46]. Using Western blot analysis of γ -H2AX, we investigated the effect of *MAP3K7* silencing on CPT-associated DNA damage. As shown in Fig. (5A), *MAP3K7* silencing appreciably enhanced γ -H2AX following CPT treatment. Moreover, the *MAP3K7* silenced cells were unable to eliminate the γ -H2AX induced by a 2 h pulse with CPT followed by a 24 h recovery (Fig. 5A), suggesting persistent DNA damage upon *MAP3K7* silencing.

The phosphorylation of H2AX (γ -H2AX) has also been linked to cellular apoptosis [47, 48]. To delineate whether the increased γ -H2AX response to CPT combined with *MAP3K7* silencing was due to enhanced apoptosis or a lack of repair, cells were co-treated with the pan-caspase inhibitor Z-VAD-FMK. As shown in Fig. (5A), Z-VAD-FMK effectively eliminated differences in γ -H2AX between siMAP3K7-3 and siNegative transfected cells indicating that TAK1 (*MAP3K7*) acts primarily by suppressing the apoptotic response to CPT.

Further investigation will be needed to explicitly determine which downstream effects of TAK1 inhibition are involved in increasing CPT-mediated apoptosis. Recently, additional mechanisms of survival have been associated with TAK1 activity. For example, TAK1 can reduce reactive oxygen species through a process independent of NF- κ B [49]. TAK1 can also inhibit the transcriptional activity of the forkhead transcription factor FOXO1 through Nemo-like kinase (NLK). This suppresses transcription of the pro-apoptotic protein Bim and cell cycle protein p27 [50]. Moreover, TAK1 can mitigate death signal from the TNF- α receptor through regulating transient phosphorylation of the epidermal growth factor receptor EGFR [51]. Regardless of the mechanism, TAK1 signaling can clearly protect cells against a variety of stresses.

TAK1 can be activated through stimulation by growth factors and cytokines. For example, the ubiquitin ligase TRAF6 can be activated by a variety of growth factors and cytokines, including IL1, resulting in subsequent recruitment of TAB2 and the activation of TAK1 [52]. Our initial screen included two siRNAs corresponding to TRAF6; however, neither sensitized CPT (Supplemental Table S1). An independent, larger CPT-siRNA screen (7,000 genes, unpublished data) that included four siRNAs corresponding to *TRAF6* did identify two *TRAF6* siRNAs that modulated the activity of CPT. A dose response analysis of in the context of TRAF6 knockdown using these siRNAs yielded sensitization comparable to that of TAK1 (MAP3K7) and TAB2 (Fig. 6). We also co-silenced *TAK1* and *TAB2*, and *TAK1* and *TRAF6* using a combination of siRNAs targeting both genes in an effort to increase sensitivity. In neither case did we see further enhancement of CPT sensitization see when each gene was silenced individually (data not shown).

Overall, this study further demonstrates the utility of RNAi screening approaches. By conducting a relatively small siRNA-based screen corresponding to approximately 400 apoptosis-related genes, we identified the inhibition of TAK1 as a significant enhancer of CPT activity in MDA-MB-231 breast cancer cells. Follow-up investigation confirmed this activity in additional cell lines, including HCT-116 colon cancer cells suggesting that this effect is independent of altered hormone receptor signaling. Further, we identified additional, related targets TAB2 and TRAF6 as proteins whose loss of function enhances CPT cytotoxicity, though an inhibitor of TAK1 kinase activity did not act synergistically with CPT. Together, our data suggests that the protective effects of TAK1 are dependent upon upstream signaling and scaffolding events and may be independent of the kinase activity of TAK1. Recent studies have also demonstrated that DNA damage, either through ionizing radiation or chemical agents, can activate TAK1 through a process dependent upon ATM and NEMO, which leads to the activation of NF- κ B [53–55]. These findings, together with our own, suggest that the inhibition of TAK1, or other, closely related members of its signaling pathway, may offer a strategy towards improving the activity of camptothecins and other DNA damaging agents, potentially broadening their application [56]. Moreover, since the inhibition of TAK1 did not sensitize all cell lines examined in this study, its inhibition may offer a way to selectively improve compound activity in a cell-type specific manner. Further studies are needed to determine which genetic backgrounds are susceptible to this type of strategy. Notably, in addition to augmenting compound activity, the targeting of TAK1 may have additional benefits. For example, the disruption of TAK1 and TAB2 has been found to contribute to the metastatic potential of breast cancer cells by reducing the expression of MMP9, a matrix metalloproteinase [57, 58]. Additionally, TAK1 was found to be required for TGF- β stimulated invasion by MCF10A-CA1a breast cancer cells [59]. Thus, the targeting of TAK1 may not only sensitize certain cells to cytotoxic agents such as CPT, but may also affect metastasis, making it a very attractive molecular target.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ATM	ataxia telangiectasia mutated
CPT	Camptothecin
H2AX	H2A histone family, member X
JNK,MAPK8	mitogen-activated protein kinase 8
MAP3K7	mitogen-activated protein kinase kinase kinase 7
NEMO	NF- κ B essential modulator
NF-κB	nuclear factor of kappa light polypeptide gene enhancer in B-cells
p38,MAPK14	mitogen-activated protein kinase 14
RNAi	RNA interference
siRNA	small interfering RNAs
TAB2	TGF-beta activated kinase 1/MAP3K7 binding protein 2
TRAF6	TNF receptor-associated factor 6
γ-H2AX	Phosphorylated H2A histone family, member X

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Fig. 1. siRNA screen and follow-up dose response analysis

A. Results from the CPT chemosensitization RNAi screen conducted in MDA-MB-231 breast cancer cells. Cells were transfected with siRNA (20 nM). After 48 h, the media was removed and fresh media containing either 200 nM CPT ($\approx EC_{50}$, 0.1% DMSO) or vehicle only (0.1% DMSO) was added and the cells incubated for an additional 48 h at 37 °C. After this time, cell viability was assayed (Cell Titer Blue Reagent, Promega, Madison, WI). The normalized activity of each siRNA in the presence of CPT was plotted versus the normalized activity of each siRNA in the absence of CPT. **B.** A Z-score plot of the screen data: Z-scores were calculated as $z = (x - \mu) / \sigma$ where x is the experimental value; μ is the median screen value and σ the standard deviation for the screen. The top sensitizing siRNA (siMAP3K7-1), with a value near 5 standard deviations from the median, corresponded to *MAP3K7*. A second siRNA corresponding to *MAP3K7* (siMAP3K7-2) also enhanced CPT cytotoxicity, although not as significantly. **C.** Follow-up dose response analysis of siMAP3K7-1 in MDA-MB-231 cells. Cells were transfected with 20 nM siRNA and CPT was added at the doses shown 48 hours later. Cell viability (Cell Titer 96 Aqueous One Solution Reagent, Promega) was assayed 72 hours after addition of CPT. **D.** Corroboration of this activity with an additional, sequence-independent siRNA targeting *MAP3K7* (siMAP3K7-3). Cell viability (Cell Titer 96 Aqueous One Solution Reagent) was assayed 72

hours after addition of CPT. **E.** Dose response analysis in MDA-MB-468 cells. MDA-MB-468 cells were transfected with 20 nM siRNA and CPT was added at the doses shown 48 hours later. Cell viability (Cell Titer Glo (Promega)) was assayed 48 hours after addition of CPT. In Figures C-E the values in parenthesis indicate 95% confidence intervals.

Fig. 2.

Examining the effect of *MAP3K7* silencing in additional cell lines and in the context of additional compounds. **A.** *MAP3K7* silencing has no effect on CPT activity in MCF10A mammary epithelial cells (left). However, activity is not restricted to MDA-MB-231 cells, as the silencing of *MAP3K7* also sensitized HCT-116 colon cancer cells to CPT (right). **B.** In addition to CPT, silencing *MAP3K7* also sensitized MDA-MB-231 cells towards topotecan (left) and doxorubicin (right). **C.** Silencing *MAP3K7* also sensitized HCT-116 cells towards topotecan (left) and irinotecan (right). Cells were transfected with 20 nM siRNA for all experiments. As with the primary screen and initial follow-up (Fig. 1), the media was removed 48 h post-transfection and fresh media containing the indicated compound was added. Viability was assayed (Cell Titer 96 Aqueous One Solution Reagent) 48 h (HCT-116) or 72 h (MDA-MB-231 and MCF10A) post-addition. Values in parenthesis indicate 95% confidence intervals.

Fig. 3.

Examining other members of the TAK1 signaling complex. **A.** Evaluating siRNAs targeting other components of the TAK1 signaling complex identified two siRNAs targeting TAB2 that sensitize MDA-MB-231 cells to CPT. **B.** Follow-up dose response analysis of siTAB2-1 and siTAB2-2 in MDA-MB-231 cells. **C.** Follow-up dose response analysis of siTAB2-1 and siTAB2-2 in HCT-116 cells. For all experiments, cells were transfected with 20 nM siRNA. Media was removed and CPT was added in fresh media. Viability was assayed (Cell Titer 96 Aqueous One Solution Reagent) 48 h (HCT-116) or 72 h (MDA-MB-231) post-addition. Values in parenthesis indicate 95% confidence intervals.

Fig. 4.

Examining apoptosis and downstream components of the TAK1 signaling pathway. **A.** siMAP3K7-3 enhanced CPT-induced caspase 3/7 activities in MDA-MB-231 cells. Cells were transfected with either MAP3K7-3 or negative control siRNA as described above. After 48 h, the media was removed and 100 μ L of fresh media containing either CPT or vehicle only (0.1% DMSO) was added. Caspase 3/7 activity was measured using the Caspase-Glo 3/7 assay (Promega, Madison, WI) at the indicated time points. **B.** EMSA analysis indicated that *MAP3K7* silencing does not appear to affect CPT-induced NF- κ B activation in MDA-MB-231 cells. Cells were transfected with siMAP3K7-3. After 48 h, cells were treated with either 10 μ M CPT or vehicle only (0.1% DMSO) 3 h at 37 $^{\circ}$ C before harvesting for EMSA analysis. **C.** Screening of siRNAs that target downstream kinases of TAK1 found none that reproducibly sensitize MDA-MB-231 cells. As indicated, these downstream kinases are associated with NF- κ B, JNK, and p38 activation.

Fig. 5.

MAP3K7 silencing increases CPT-associated γ -H2AX that is eliminated by treatment with the pan caspase inhibitor Z-VAD-FMK (100 μ M). Cells were transfected with siMAP3K7-3 (20 nM) and either 1 μ M CPT or vehicle only (0.1% DMSO) was added 48 h post-transfection. Cells were incubated for an additional 24 h at 37 °C before lysing for Western blot analysis, or incubated for 2 h before replacement of the CPT-containing media with fresh media. Cells undergoing media replacement were incubated for an additional 24 h in fresh media. For Z-VAD-FMK experiments, cells were incubated with 100 μ M Z-VAD-FMK (0.1% DMSO) for 2h prior to CPT addition and maintained in Z-VAD-FMK until harvesting for Western blot.

Fig. 6.

The silencing of TRAF6 yields sensitization comparable to that of TAK1 and TAB2. This suggests that the protective effects of TAK1 are dependent upon upstream signaling and scaffolding events. MDA-MB-231 cells were transfected with 20 nM siRNA for 48 h. CPT was then added and viability was assessed after 72 h.