Phenothiazine Inhibitors of TLKs Affect Double-Strand Break Repair and DNA Damage Response Recovery and Potentiate Tumor Killing with Radiomimetic Therapy

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Abstract

The Touseled-like kinases (TLKs) are involved in chromatin assembly, DNA repair, and transcription. Two TLK genes exist in humans, and their expression is often dysregulated in cancer. TLKs phosphorylate Asf1 and Rad9, regulating double-strand break (DSB) repair and the DNA damage response (DDR). TLKs maintain genomic stability and are important therapeutic intervention targets. We identified specific inhibitors of TLKs from several compound libraries, some of which belong to the family of phenothiazine antipsychotics. The inhibitors prevented the TLK-mediated phosphorylation of Rad9(S328) and impaired checkpoint recovery and DSB repair. The inhibitor thioridazine (THD) potentiated tumor killing with chemotherapy and also had activity alone. Staining for γ -H2AX revealed few positive cells in untreated tumors, but large numbers in mice treated with low doxorubicin or THD alone, possibly the result of the accumulation of DSBs that are not promptly repaired as they may occur in the harsh tumor growth environment.

Keywords

inhibitors of Touseled kinases, radiomimetic sensitizers, mechanism of DSB repair, DNA damage response

Introduction

A high percentage of human tumors, including cancer of the prostate (CaP) and breast (BCA), show mutations in DNA repair genes and checkpoint functions that make them overly dependent on alternative pathways for survival. Unfortunately, this can result in carcinomas that are highly resistant to radiation therapy (XRT) or radiomimetic therapy (RMT) from fail-safe repair mechanisms also designed to contain excessive genomic instability. Targeting those mechanisms can result in highly specific and effective therapies. Clearly, some success from the adjuvant use of PARP inhibitors to treat BRCA mutants and/or triple-negative BCA¹ and other strategies to exploit DNA repair defects point in that direction.² We propose that the addition of newly identified inhibitors of Touseled-like kinases (TLKs) to enhance the response to XRT/RMT will greatly benefit the management of CaP and BCA patients. In fact, ameliorating the adverse effects of standard therapy, by reducing the doses while maintaining specific cancer killing, still seems to be one of the most promising courses of action for the near future.

The TLKs are involved in chromatin assembly, DNA repair, transcription, and chromosome segregation.³ Two TLK genes (TLK1 and TLK2) with several splice variants

were identified in humans. TLK1/1B interacts specifically with the chromatin assembly factor Asf1 and Rad9,⁴⁻⁶ and we have presented evidence that TLK1B promotes repair

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by processing the double-strand break (DSB) ends and disassembling chromatin nearby to facilitate the recruitment of repair proteins.⁴ Since Rad9 is a critical mediator of the DNA damage response (DDR) and in repair (specifically of DSBs), it seemed that the TLK1-Rad9 interaction would be very important in implementing TLK1B-mediated radio-protection. The past few years have witnessed significant advances in understanding the roles of TLKs in the DDR⁷ and in DSB repair⁴ as well as their clinical relevance. In BCA, elevated expression of the TLK1B splice form is found in approximately 30% of the patients and often corresponds to poor response to XRT and doxorubicin (doxo),⁸ presumably due to efficient DSB repair in the tumor cells. We postulated that its expression could serve as a marker for prognosis as well as a target for therapeutic intervention. In addition, there are BCA cases in which TLK1/1B is not elevated, but TLK2 is amplified and/or overexpressed.^{9,10} Thus, for a large proportion of sporadic BCA, specific TLK inhibitors should be extremely beneficial as radiosensitizers and chemosensitizers. The fact that TLKs are overexpressed likely renders tumor cells more dependent on these kinases than normal tissues and hence their preferential TLK-targeted killing. In contrast to BCA, in the most common human CaP cell lines, only one or the other TLK gene is expressed, although typically at high levels¹¹; we do not have the story yet for the analysis of patient samples. If this represents a typical situation for CaP, then such cells would be even more dependent on the expression of either TLK and hence their preferential killing with an inhibitor.

In this work, we report the identification of some specific inhibitors of TLK, and surprisingly, some of these belong to the family of phenothiazine antipsychotics that have been approved for the treatment of schizophrenia for years. Consistent with the proposed role of TLK in mediating DSB repair, only the phenothiazines that inhibit TLK sensitized cell killing with RMT. The inhibitors specifically inhibited the TLK-mediated phosphorylation of Rad9(S328) that is DDR responsive and impaired recovery from the checkpoint. We further established that the inhibitors act specifically on repair pathways controlled by TLK and result in specific inhibition of nonhomologous end joining (NHEJ) in several assays. Further evidence for their high specificity stems from the fact that only some of phenothiazine antipsychotics were inhibitory, while others that are structurally very similar were not, and only those that inhibit TLK sensitized cell killing with RMT (and radiation [IR]). Of note, this is not the first time that some phenothiazines have been shown to inhibit DSB repair and enhance killing of cancer cells in combination with RMT.¹²⁻¹⁴ However, identification of the molecular/cellular target (TLKs) for the sensitizing effects observed has remained elusive. In these previous reports, the investigators were aware that the target was not known and hence would fail to

provide critical intermediate markers (like P-Rad9(S328)) to monitor the effectiveness of the drugs/therapy in future clinical trials. Some investigators proposed DNA-PK as the target for RMT sensitization¹⁵ or calmodulin.¹⁶ In reality, neither of these pass scrutiny because phenothiazines inhibit these proteins *in vitro* only at concentrations >50 μ M, which makes these 2 targets implausible, first, because the RMT sensitization effect in cultured cells is obvious at low (μ M) doses, and second, at concentrations >20 μ M, these drugs are nonspecifically toxic and cannot be tested in combination with RMT. We report now that some antipsychotic phenothiazines specifically inhibit TLK at (nM) concentrations *in vitro* and at low, well-tolerated (μ M) concentrations in cultured cell lines. We further establish that the inhibitors act specifically on the DSB repair pathways controlled by TLKs. Xenograft studies in SCID/bg mice harboring PC-3 human CaP cells or MDA-231-Luc human BCA cells were undertaken to test the *in vivo* effects of one selected TLK inhibitor, thioridazine (THD), with or without doxo, and showed synthetic effects.

Much work in cancer therapy is devoted to the problem of drug resistance. However, resistance is a late outcome that could be avoided by the implementation of better combination therapies to reduce the RMT doses while still specifically targeting cancer cells. We have confirmed previous work that some, but not all, phenothiazine antipsychotics inhibit DSB repair and potentiate tumor cell killing with XRT and RMT and in fact can have some antitumor properties by themselves *in vivo* due to the harsh tumor environment, eliciting the formation of DSBs. We also have demonstrated that the main molecular/cellular targets of these drugs, in relation to their effect on the inhibition of DNA repair and RMT potentiation, are the TLKs. The identification of phenothiazine antipsychotics as inhibitors of DSB repair and the potential consequence for the suppression of cancer emergence in patients treated for schizophrenia are intriguing, and there is some interesting literature on the subject.¹⁷

Results

Identification of TLK Inhibitors: Inhibition of TLK1B Autophosphorylation by Selected Phenothiazines. To identify inhibitors of TLK, we developed a high-throughput screen with recombinant TLK1B, a small Rad9 peptide, and the ADP-Hunter reagent (DiscoverX, Fremont, CA). Using this assay, the Innovative North Louisiana Experimental Therapeutics (INLET) screening core (affiliated with the Feist-Weiller Cancer Center) screened the Prestwick library, 2 other proprietary libraries, and a subset of the ChemDiv library (~6,000 compounds). We soon identified 4 inhibitors that are structurally and chemically similar and belong to the class of phenothiazine antipsychotics, although some

Figure 1. Inhibition of TLK1/IB autophosphorylation by phenothiazines. **(A)** Selected phenothiazines were tested in autokinase assays employing $\gamma^{32}\text{P}$ -ATP and determined by TCA-precipitable counts (% inhibition compared to no drug). **(B)** The TLK1/IB inhibitors were tested in cultured cells by IP/autokinase/autoradiography. 293T cells were incubated for 1 hour with TLK1/IB inhibitors (TFP and PPH). Extracts were prepared for IP as described in Materials and Methods, followed by autophosphorylation with $\gamma^{32}\text{P}$ -ATP. The blots were then probed with TLK1 antiserum.

similar drugs in the same family were inactive (table in Suppl. Fig. S1).

Following the initial fluorescent screen at a compound concentration of approximately 5 μM , potential hits were tested with a more sensitive autokinase assay with $\gamma^{32}\text{P}$ -ATP. Inhibition of autophosphorylation was confirmed by TCA-precipitable counts (Fig. 1A). The inhibitors are highly specific for TLKs. A commissioned KinomeScan (DiscoverX; see link for list: <http://www.discoverx.com/services/drug-discovery-development-services/kinase-profiling/kinomescan>) with THD revealed that no other kinase in the panel was significantly inhibited (>60% at 10 μM), and the compounds do not resemble ATP (the kinase assay dataset is available upon request for THD and PPH). Figure 1B shows that the drugs worked specifically at low (μM) concentration after immunoprecipitation (IP) of TLK1 from 293T cells, and interestingly, the drugs remained associated with the protein, retaining their inhibition even after removal from the medium; additional drugs were not added to the IP in the kinase reaction. We have additional evidence for direct binding of these compounds to the recombinant protein (to be published elsewhere).

The TLK Inhibitors Delay DSB Repair: Kinetics of Repair in Episomes Containing a Single HO-Generated DSB, and Slower Dissolution of γ -H2AX Foci Induced by Bleocin Treatment. We have used several assays to study DSB repair. In initial studies, the inhibitors delayed the repair kinetics of the single DSB introduced with Ad-HO⁴ on a novel multicopy episomal repair system (Fig. 2A). This system offers the advantage of generating 50 to 100 DSBs per cell that are all equivalent, genetically and in end type, and in their chromatin context. The repair appears to be primarily accurate NHEJ (simple plasmid reclosure) for these episomes, as we have not yet obtained evidence of the formation of concatemers, even when the population of plasmids was not completely cut, thus offering intact template strands for HRR between plasmid molecules and presumably resulting in Holliday junctions and concatemeric units that we were unable to detect on Southern blots (work to be published elsewhere). Note also that this episomal system is fully capable of recapitulating the DDR induced by multiple genomic DSBs in mamma-

lian cells, such as activation of ATM, Chk1-mediated inhibitory phosphorylation of TLK1(S695), and generation of γ -H2AX (Fig. 2B) most likely on the episomal chromatin itself, just as was reported for plasmids replicating in yeast.¹⁸

The inhibitors also caused markedly increased sensitivity to IR or Bleocin (Calbiochem, San Diego, CA [Cat. No. 203408]) that could be explained by the inhibition of DSB repair. This was shown as slower regression of DSB repair foci (γ -H2AX) (Fig. 3), which shows a representation of the main features we observed after DSB induction, namely that there were generally more foci per cell, they were brighter, and they persisted for longer periods in the presence of TLK inhibitors.

Repair of a DSB In Vitro. We have developed a system using nuclear extracts of MM3MG cells that allows us to monitor the repair of a plasmid cut with EcoRI, which leaves a cohesive 5' overhang.^{5,19} Ligation of the ends and circularization of the plasmid, concomitant with chromatin formation, result in the introduction of negative supercoils. Similar

Figure 2. Kinetics of repair in episomes. **(A)** Kinetics of repair in episomes cleaved with adeno-HO endonuclease (Ad-HO) during a time course of infection. 293T cells were treated (or not) with TFP or PPH 12 hours prior to infection. Kinetics of repair was followed by qPCR with primers flanking the HO cut site, at the pRSV promoter and at the CAT gene. Data points were normalized for an amplicon in the Amp gene and also for a genomic site to ensure the isolation of equal amounts of DNA. **(B)** Infection with Ad-HO and generation of a single DSB in episomes result in the phosphorylation of ATM (activation), TLK1(S695) (inhibition), and H2AX(S139). Infection of 293T cells that do not contain the HO-targeted episomes does not result in sufficient ATM activation (bottom panel).

systems were described for the stimulation of chromatin assembly by the histone chaperone CAF1.²⁰ We showed that the addition of TLK1B hastened the repair and supercoiling of the plasmid⁵ with some involvement of Asf1^{21,22}; formation of chromatin in such assays is confirmed by analyses of regularly spaced nucleosomal ladders with MNase. While initial assays dealt with the ligation of cohesive ends, we subsequently tested the ligation of incompatible ends (more representative of damaged ends typically obtained

with XRT or RMT) that can be ligated only after some processing with a Rad9 dependency.⁵ Successful end joining and simultaneous plasmid supercoiling were monitored as an increased mobility (Fig. 4). Transformation of bacteria and analysis of the repaired junctions revealed a mix of fill-in and resection of the ends.⁵ In new experiments here, a panel of TLK inhibitors reduced end filling and repair/religation with a Rad9 dependency (example in Fig. 4), indicating that the inhibitors also have a direct effect on DSB repair. Notably, 9-1-1 is known to recruit repair polymerases at incompatible ends to promote filling,²³ which must precede ligation/supercoiling as only closed plasmids can be supercoiled.⁵

Inhibition of Rad9 Phosphorylation.

The inhibitors worked as expected on the pattern of phosphorylation of Rad9(S328). After treatment of DU145 cells with H₂O₂ for 10 minutes to generate breaks, there is a corresponding wave of phosphorylation and dephosphorylation of S328 after completion of the repair (Fig. 5) that matches the pattern of activity of TLK1/1B; TLKs are the only known kinases that phosphorylate Rad9(S328).⁴ Generation of DSBs causes transient inhibition of TLK1 and simultaneous synthesis of TLK1B, which then results in hyperphosphorylation of S328 upon the restoration of kinase activity (Fig. 5A).⁵ Once H₂O₂ is degraded and most DNA breaks are repaired, the phosphorylation returns to baseline.

The inhibitors, PPH (LS125) in particular, blocked the activation of TLK and consequent phosphorylation of Rad9(S328) (Fig. 5C). This resulted in persistent activation of Chk1 (P-Chk1(S345)) (Fig. 5D), which in turn resulted in prolonged phosphorylation of Rad17 (Fig. 5E) and a block in the release of the RFC/Rad17/9-1-1 clamp loader/clamp complex at the repaired junctions (see model in Suppl. Fig. S3) and previous results of ChIP analysis in cells expressing KD-TLK.⁵ This leads to a failure to deactivate the checkpoint and consequently

Figure 3. Presence of DSB repair foci (γ -H2AX foci) after DSB induction in DU145 cells. **(A)** DSBs were induced by 3 μ M of Bleocin (Calbiochem, San Diego, CA [Cat. No. 203408]). Cells were treated with 3 μ M Bleocin and +/- 10 μ M TFP for 24 hours. After 24 hours cells were washed with 1XPBS and fresh media was added. Cells were allowed to recover for 0, 2, and 4 hours respectively at 37°C. γ -H2AX (red) signal was captured using a Zeiss Axioskop microscope after 0, 2 and 4 hours of recovery time. **(B)** Percentage of cells with $\geq$ the indicated number of γ -H2AX foci, at least 150 cells per treatment, and time point were counted and grouped into tertiles; error bars were not added since this analysis is largely qualitative, unless thousands of cells can be accurately scored.

increased cell death.⁵ In fact, cleavage of cdc25A, a target of Chk1 and a key regulator of cdk2 and cdk4, is elicited much faster in the presence of LS125 (Fig. 5F), further explaining the rapid and prolonged cell cycle arrest. Similar experiments with doxo gave very similar results (Suppl. Fig. S4). A key role of TLKs is then to participate in the restoration of the cell cycle upon completion of the repair. Interfering with this process has dire consequences for cell viability, more likely for cancer cells that are genetically more unstable and already have defects in DNA repair functions, if not just for their reliance on these kinases.

Effects on Cell Cycle and Viability: RMT Potentiation In Vitro. The TLK inhibitors (e.g., TFP-LS392) even at 15 μ M have

little effect on the cell cycle or other deleterious effects, as shown by a very modest increase in (apoptotic) sub-G1 cells, with very modest evidence of cleaved PARP (Suppl. Fig. S2). In contrast, when combined with doxo, the inhibitors caused massive apoptosis and a partial S-phase arrest, which coincidentally corresponds to the cell cycle phase when TLK activity is maximal.⁶ A matching experimental set was processed by Western blot (WB) for evidence of cleaved PARP and possible effects on ClnD1 accumulation, which occurs in late G1 and throughout the S phase. Indeed, concomitant treatment of doxo + LS392 caused a maximal increase in ClnD1 levels, and corresponding with the apoptotic profile, PARP cleavage was highest in this treatment group (Suppl. Fig. S2, inset). The inhibitors had generally similar potency in additive killing with doxo in both DU145 and PC-3 (Fig. 6). The RMT potentiation is not limited to these 2 CaP cell lines but is also true for MDA-MB231 BCA cells (Fig. 6). For this experiment, we use MDA-MB231-Luc cells, so that viability could be measured directly by luminescence, which is directly proportional to the number of cells and their health and does not rely on viability dyes that can reach signal saturation; chemoluminescence in these cells is linear over several orders of magnitude. The TLK inhibitors by themselves had little effect on MDA-231 viability and doubling (Suppl. Fig. S5). The low toxicity of these

TLK inhibitors is evident in the dose response in which increasing the drug concentrations up to 15 μ M had only a modest effect on viability (Fig. 6), whereas a significant increase in cell killing was observed with increasing doxo or bleomycin and the TLK inhibitors at 5 μ M (Fig. 6). This is consistent with their proposed mechanism as inhibitors of DSB repair. In a larger panel of cell lines, the TLK phenothiazine inhibitors did not affect doubling in most cells, but there was slight inhibition in a few cell lines (Suppl. Fig. S5).

Animal Studies

PC-3 model. A study was undertaken to investigate the *in vivo* efficacy of THD compared to doxo and to determine

Figure 4. Repair of a DSB *in vitro*: ligation of EcoRI/EcoRV-cut plasmid. The effect of TFP on incompatible end filling and ligation/supercoiling and Rad9 supplementation. The top and bottom panels (EtBr and autorad) show the time course of plasmid religation/supercoiling, which includes filling of the EcoRI site with radiolabeled dATP (5 μ Ci [α - 32 P] dATP/0.1 mM) by endogenous polymerases. Ligation of the ends and assembly of chromatin are shown by the accumulation of faster mobility topoisomeric forms; these are the result of nucleosome-driven formation of negative supercoils.^{5,22} The EtBr band of highest compaction (s.c.II) was graphically quantitated below each lane with ImageJ software (National Institutes of Health).

any sensitization conferred to the tumor cells by treatment with a combination of the 2 drugs. Eighty male SCID/bg mice received 1×10^6 PC-3 cells (human CaP cells) subcutaneously into their flanks. At day 49 after inoculation, we began weekly tumor measurements with a caliper (volume = $[L \times W^2]/0.52$). The PC-3 model in our hands resulted in slow-growing tumors without the typical exponential phase that is optimal to support a case for growth inhibition in treated groups. There was no difference in tumor size between all groups ($n = 9$ each) at the initiation of the treatments. At day 54, daily oral gavages of THD (0.25 mg in water) were given, and intraperitoneal injections of doxo (1 mg/kg) (see arrows in Fig. 7) were given at days 54, 68, and 95 (last day before euthanasia). In one arm of the study,

control group tumors reached the preset end point of 1,500 mm³) and processed for immunohistochemistry (IHC) analysis. The mice were dosed on the last day before euthanasia with doxo to induce *bona fide* DSBs.

IHC Results. A composite figure that is representative of many slides from tumors from 5 mice from each group is shown in Supplementary Figure S6. Some features are immediately noticeable even upon H&E staining that could explain the tumor growth patterns. It was immediately obvious that, while treatment with doxo only slightly decreased the cellular density of the tumor, treatment with THD clearly reduced the tumor density, with fewer tumor cells and more fibrous interstitial tissue/stroma. Staining for

5- and 10-mg/kg doses of doxo (with and without THD) were administered, but these published doses proved to be too toxic. There was a statistically significant difference in tumor growth only at day 79 and day 86 for the treated groups ($P < 0.05$ and 0.02, respectively). However, a definite trend was seen for doxo-reducing tumor growth at subcurative doses. Most interestingly, THD had a similar trend of tumor growth inhibition with doxo, a standard of chemotherapy, but without any toxicity or behavioral changes. In this experiment, the combination of THD and low doxo did not prove to be statistically more efficacious than either drug alone, although there could be an “additive” trend that is more difficult to prove for statistical significance, but the fact that THD was already almost as effective as low doxo in controlling tumor progression was a main cause for the apparent lack of the synthetic effects that we instead observed for PC-3 cells in culture. There is a curve inflection following the second dose of doxo for both groups (+/- THD), and considering that the dose and schedule of doxo administration was not fully optimized, we concluded that perhaps a more quantitative model (MDA-231-Luc cells) to more precisely measure tumor growth could yield more definitive results. In any case, the tumors were excised at the end of the experiment (before the

Figure 5. The TLK inhibitors block the phosphorylation of Rad9(S328) and impair the deactivation of the DDR. **(A-C)** DUI45 cells were incubated with TLK inhibitors (PPH-LS125 or THD-LS078) for 2 hours and then with H₂O₂. A sample without H₂O₂ DSB induction is shown in lanes 8 and 12. **(D)** Chk1 remains activated (phosphorylated) after the recovery from H₂O₂ in cells treated with inhibitors. **(E)** Rad17(S645) remains phosphorylated in the presence of LS125 and LS078, which alone do not result in Rad17 phosphorylation (not shown). **(F)** Cleavage of CDC25A and release of the 33-kDa product,⁵⁹ consequent to Chk1 activation, which are expected to result in a quicker and prolonged cell cycle arrest (as was shown in Suppl. Fig. S2).

Figure 6. TLK inhibitors potentiate RMT killing in DU145 PC-3 and MDA-MB231-Luc cells. **(A, B)** Viability of DU145 and PC-3 cells with varying concentrations of doxo + TFP, THD, or PPH (10 μM) added 12 hours before the addition of doxo. **(C, D)** Viability with various concentrations of phenothiazines only. Viability was measured after 24 hours with CellTiter 96 Aqueous One Solution Cell Proliferation (Promega). **(E)** Viability of MDA-MB231-Luc cells. Treated and control cells were assessed with luminescence. RLU values were measured, with the Promega Luciferase Kit, as a direct viability indicator with DSBs induced with increasing Bleocin (Calbiochem, San Diego, CA [Cat. No. 203408]).

Figure 7. Tumor growth in mice. Xenograft assays (10^6 cells injected s.c.) were used to assess the effect of THD and doxo in the growth of CaP tumors, PC-3, in male mice or MDA-MB231-Luc in females. Tumor size was monitored twice weekly with calipers and by *in vivo* imaging. Groups included control, THD and doxo, and THD + doxo.

γ -H2AX revealed that few cells were positive in the untreated tumors, but there was a large proportion in mice treated with low doxo. Most importantly, there was a large proportion of positive-staining cells also in tumors from mice treated with THD alone. We interpret this as being the result of the accumulation of DNA damage, including DSBs, which are not promptly repaired, even as they may be expected to occur spontaneously in many cells in the harsh environment of tumor growth. This has in fact been reported before and was attributed to localized hypoxia.²⁴ As anticipated, the combination of doxo + THD gave the highest density of γ -H2AX-positive cells and also the strongest signal per cell, indicative of larger numbers of unrepaired foci. Staining for PCNA revealed a pattern consistent with the previous observations. In controls, which

showed the highest level of cellularity, a majority of the cells were clearly proliferative and stained strongly for PCNA. Tumors from mice treated with low doxo alone showed a similar pattern and percentage of PCNA-positive cells but perhaps a slightly lower cell density. In mice treated with THD alone, there were significantly fewer PCNA-positive cells, consistent with the pattern of lesser cellularity. Nonetheless, the staining was intense in individual nuclei, indicating that the density of replication forks was not affected in those cells that were replicating, and hence, the proliferation rate was probably normal. In contrast, the PCNA-positive cells in tumors of mice treated with doxo + THD were not only fewer in number but were also much lighter. This was observed in all slides that we inspected for this group of mice. The staining was not negative, as one may think, since inspection at a higher magnification confirmed the expected pattern of exclusive nuclear staining and lower intensity in nucleoli. It suggested instead a lower density of PCNA per nuclei, which would be expected from cells harboring stalled replication forks and replicating more slowly, and consistent with our observation, the aforementioned study on hypoxic tumor areas reported an inverse pattern of staining for Ki67 (very weak) in which γ -H2AX-positive cells were

located.²⁴ This is consistent with the γ -H2AX pattern, showing a large number of cells harboring repair foci, and thus likely arrested in replication (or progressing slowly through the S phase). To further establish this, we looked at the presence of RPA foci, which is indicative of stalled forks.²⁵ As expected from the large number of proliferating cells (PCNA positive) in control tumors, RPA1 staining was very strong and particularly in focal areas. RPA1 staining in tumors from doxo-treated mice was interesting. The overall staining was weaker for most areas of the section, consistent with the idea that we propose that cell proliferation is hampered by the need to repair DSBs. But interestingly, there were also areas of more intensely stained cells localized to necrotic spaces, which could be explained by the additional need for RPA during DNA repair in instances of

damage that results in the extensive formation of ssDNA,²⁶ possibly in cells in the most hypoxic areas of the tumor. RPA staining in tumors from THD-treated mice was similar to those treated with doxo, but in contrast, the staining in necrotic areas was weaker. In tumors from mice treated with doxo + THD, staining for RPA was very weak and consistent to that obtained for PCNA. We did not probe for the presence of apoptotic cells (TUNEL), which are rarely found *in vivo* in any case. However, we did stain for P-Rad9(S328) to confirm that the effects were mediated via TLK inhibition, in what we propose is part of a checkpoint deactivation mechanism following repair of spontaneous DNA damage. Indeed, numerous cells stained lightly for P-Rad9 in untreated tumor cells; in fact, they are perhaps a larger proportion than those staining for γ -H2AX that presumably represent cells with unrepaired DSBs. Cells in tumors from animals treated with doxo showed much stronger staining for P-Rad9, and perhaps the same density as those staining for γ -H2AX, suggesting that chemical induction of DSBs and repair occur concomitantly. Treatment with THD resulted in the severe suppression of P-Rad9, despite clear evidence for the presence of γ -H2AX in the same tumors (hence, DSBs). Similarly, treatment with doxo + THD severely depressed P-Rad9, which we ultimately interpret as an incapacity to complete DSB repair and recovery from the DDR checkpoint.

MDA-231–Luc Model. We used the BCA model cell line MDA-231–Luc for more precise quantitation of tumor growth (photon emission is linear with the tumor cell number). Female SCID/bg mice ($n = 8$ per group) received 1×10^6 cells, and when the tumors reached approximately 500 mm³ ($\sim 10^4$ photons/sec), they were assigned to 4 treatment groups, as in the study above (Suppl. Fig. S6). With this model, which showed typical exponential growth for the control group, THD alone was effective at controlling the tumor increase and actually was better than low doxo. In this experiment, a second course of doxo in the THD-treated animals caused rapid and almost complete shrinkage of the tumors. In contrast, treatment with doxo alone was insufficient to stop the growth of tumors, although there was a curve inflection at day 25 (10 days after doxo administration) that could be due to a delayed effect of doxo on either the tumors or the animals (not necessarily linked to the accumulation of DSBs in tumor cells).

Only Some Phenothiazine Antipsychotics Available Are TLK Inhibitors and Inhibit DSB Repair. A detailed structural basis to explain why some phenothiazine compounds inhibit TLK, whereas others very similar do not, is unclear. However, an explanation may reside in quantum mechanics (QM) and electron density maps (Suppl. Fig. S1). Obviously, the electron distribution will determine how the drugs interact in a

specific fashion with particular side chains in the kinase active site. To determine how structurally related antipsychotic drugs might differ from each other and produce different biological effects, we analyzed optimized drug structures by QM methods. Drugs of interest were built with MarvinSketch 5.3.2 (ChemAxon, Budapest, Hungary) for initial optimization of the 3-dimensional structure. For the panels shown here (Suppl. Fig. S1), orbitals from the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) have been depicted. We suggest that the binding site has very precise requirements and that it may overlap with the ATP binding site. The crystal structure of the related kinase, ASK1, shows staurosporine bound at this site.²⁷ The planar heterocyclic ring structure of staurosporine and its out-of-plane animated glucose moiety are reminiscent of the structure of the phenothiazines. Bulky substituents on the main phenothiazine ring, such as fluorine groups, may only be tolerated in a certain orientation relative to the alkyl side chain, depending upon the reactivity of the side chain. Moreover, the fit at the binding site will be determined by whether active groups in the drugs (rings v. side chains) serve as donors or acceptors in nonbond interactions (e.g., π -bonding and van der Waals interactions). While QM characterization of the drugs is not definitive, certain trends emerged from this analysis. In Supplementary Figure S1, three fourths of the phenothiazines with biological activity have a greater concentration of HOMO in the heterocyclic ring structure, whereas none of the 4 inactive compounds depicted do. When HOMO is localized in the alkyl substituent of the active drugs (e.g., TFP), it has the opposite phase of the inactive molecules (e.g., PCP). Based on these initial data, we suggest that the reactivity, or electron charge distribution, of the phenothiazine ring is an important determinant of the activity in TLK assays. Conversely, greater reactivity of frontier orbitals in the alkyl substituents of the inactive drugs may promote the interaction of these groups with TLK side chains that interferes with insertion of the phenothiazine ring into the narrow cleft at the ATP binding site. Because the precise features of the ATP binding site have been dramatically shaped by evolution, this site may be highly selective for drugs that only have slight differences in their 2-dimensional structures. Together, this information may aid the design of more potent drugs in the future. Note also that our definition of “inactive compounds” is limited to concentrations $<10 \mu\text{M}$; we make no claims above that concentration, either in TLK inhibition or for other cellular effects, especially since undue toxicity is then observed. We should add that the parental base compound, phenothiazine, is highly inhibitory and so is the dye, methylene blue. Thus, the nitrogen alkyl substituents, which are critical for the dopaminergic effects, do not appear to matter for the inhibition of TLK, although they could partly explain the lack of

it. In fact, methylene blue, which stains nucleic acids but not most proteins, remains so tightly bound to GST-TLK1B that after its affixation to an Immobilon filter (Immobilon-P, Cat# IPVH00010, Millipore, Billerica, MA, USA), the color bound to the protein will not wash out. While it is unclear whether the active phenothiazines bind an allosteric site or at/near the ATP pocket, we do know that they do not prevent the binding of the Rad9 peptide, as we have done several experiments with a fluorescein-tagged version of it. Another fact is clear: that the dopamine receptor (DR), in the unlikely case it was expressed in the cell lines that we have studied here, plays little to no role in the effects that we have reported. For instance, FF (inactive for TLK) is a more potent D2 receptor antagonist than either TFP (active) or THD (active), while PMZ does not have much activity as a D2 antagonist at all. Therefore, we find no obvious correlation between D2R antagonism and biological effects in our assays. This conclusion was also reached by Kau *et al.*²⁸ during a chemical genetic screen identifying inhibitors of regulated nuclear exports of a Forkhead transcription factor in PTEN-deficient tumor cells. They reported that a few phenothiazine antipsychotics blocked FOXO1a export in 786-O cells and with similar potency (in contrast to our results on TLK activity). After initially raising the possibility that FOXO1a nuclear relocalization might be regulated by DR signaling, they clearly demonstrated that this in fact was not the case and instead implicated calmodulin as a possible target.²⁸ We should unfortunately emphasize that their screens were carried out at 40 μ M, and later, none of their assays was carried out below 20 μ M. We have already pointed out that at those concentrations, these compounds are toxic to most mammalian cells, and hence, any potential mechanistic conclusion on the redistribution of FOXO1a export due to these compounds is dubious, and the implication of calmodulin suffers again from the fact that the protein requires concentrations >50 μ M for inhibition *in vitro*, let alone in cells.

Discussion

TLKs and Rad9. The TLKs are highly conserved proteins in animal and vegetal kingdoms, with dual chaperone kinase functions that affect several processes of chromatin assembly.³ Only a few direct “interacting” substrates of TLKs have been identified, the principals being the histone chaperone Asf1, histone H3, and Rad9. This suggested that TLKs function in processes of chromatin assembly^{4,29} that have been identified so far in transcription,^{30,31} DNA repair,^{4,19,32} replication,³³ and mitotic condensation of chromosomes.^{34,35} The human homolog, TLK1B, has invoked interest because of its established role in cell survival after DNA damage.^{19,32,36} In mammals, the primary TLK1 transcript is alternatively spliced in 2 main isoforms, TLK1 and

TLK1B³⁶; TLK1B is subject to translational regulation, and its synthesis is induced by DNA damage. Elevated expression of TLK1B promotes cell survival after IR or doxo by facilitating DNA repair,¹⁹ while expression of a kinase-dead mutant renders mammalian cells sensitive to IR.³⁴ Evidence also exists for a link between TLKs and a DNA damage relay⁷ that leads to transient kinase inhibition mediated by ATM via Chk1 by direct phosphorylation of TLK1 at S695.³³ These findings identified a functional cooperation between ATM and Chk1 in the propagation of a checkpoint response mediated by transient inhibition of TLK1, which may regulate processes involved in chromatin remodeling after damage⁷ as well as assembly of repair components at damage sites and other aspects of DDR and recovery.^{4,5}

Rad9, Rad1, and Hus1 form a trimeric complex (termed 9-1-1) that is structurally similar to the PCNA “sliding clamp,” which encircles the DNA conferring processivity to polymerases and performing many other functions, including mediation of the checkpoint.^{37,38} 9-1-1 assembles at sites of damage via the genotoxin-activated RFC-Rad17 “clamp loader.”³⁹ The 9-1-1 complex may then serve as a scaffold for the assembly of DNA repair proteins as well as engaging components for the DDR. We showed that TLK1B uniquely phosphorylates Rad9 at S328 and that this appears to play a key role in resumption of the cell cycle. However, TLK1B also had a function as a chaperone for Rad9 assembly at DSBs that was independent of its kinase function.⁴ We proposed that the regulated binding of 9-1-1 and TLK1B to DSBs recruits repair enzymes and a chromatin disassembly apparatus to promote efficient repair, and only subsequently, TLKs participate in DDR disengagement and deactivation of the checkpoint,⁵ for which we have now presented additional evidence. A model for the role of S328 phosphorylation in the release of 9-1-1 and consequent deactivation of the checkpoint is included (Suppl. Fig. S7). Recent studies showcase the importance of the ATM-dependent phosphorylation of Rad9(S272) in the maintenance of genomic stability.⁴⁰ Here, we show for the first time a critical role for the phosphorylation of S328 by TLKs in checkpoint recovery from the DDR and possibly directly in end repair *in vitro* with a plasmid system (Fig. 4). This extends our previous studies with ES-rad9^{-/-} reconstituted with hRad9(S328A)⁴ and the use of TLK1B-KD-expressing cells engineered to contain a single HO-mediated DSB.⁵ Since Rad9 is a critical mediator of the DDR checkpoint and in repair (specifically of DSBs), we proposed that the TLK1-Rad9 interaction would be very important in implementing the mechanism of TLK1B-mediated radioprotection.¹¹ The significance of the TLK-Rad9 axis is clear for CaP for which several studies have implicated Rad9’s critical role in disease progression and prognosis.^{41,42} Nonetheless,

elevated Rad9 expression, and some of its phosphorylated forms, has been noted also in BCA and correlated to prognosis.⁴³

TLKs Are Targets for Therapeutic Intervention. The TLKs are becoming the center of much attention for their role in DSB repair^{4,19,44} and their potential contribution to cancers refractory to XRT or RMT, including cholangiocarcinoma,⁴⁵ BCA,⁸ and CaP.¹¹ Note, however, that probably most technologies directed at globally reducing the expression of TLKs are unlikely to result in effective and safe therapies. Silencing RNA-mediated suppression of TLKs eventually kills all cells (normal or cancer) even without IR or RMT.^{11,30} These are essential proteins as chaperones, while the kinase activity seems less critical and needed for more complex and specialized functions, as shown here. In fact, we could produce a stable cell line of normal mammary fibroblasts expressing a TLK1-KD mutant (in the presence of wild-type endogenous TLKs), but we also used siRNA to complement the work, which arrested the cells and then killed them.³⁴ Since the mechanism of action of TLKs in the resistance to genotoxins via DNA repair is now fairly well elucidated,³ it would seem that adding TLK inhibitors could significantly improve standard therapy. While our screen for inhibitors of TLK is still ongoing, the fact that the first attempt has identified a panel of phenothiazine antipsychotics with >30 years of clinical use immediately dictated a point to stop and take stock. And indeed, we further looked at the full panel of commonly used phenothiazine antipsychotics, and only a subset had inhibitory activity on TLK and DSB repair *in vitro* and in cells. Hence, while this is not the first report that show that some of these compounds enhance cell killing with XRT or RMT via inhibition of DSB repair,¹² the proposed generalization that all these drugs can do so is incorrect.¹³ Moreover, none of the previous studies identified the correct molecular/cellular target(s) for previously noted reasons in regard to effective doses. We can clearly exclude the involvement of DNA-PK for several reasons. First, at subtoxic concentrations of TFP (10-20 μ M) that completely inhibit TLKs, DNA-PK is not inhibited (reported inhibition *in vitro*: 0.1-0.2 mM).¹⁵ Second, the phenothiazine concentrations used in that study induced strong apoptotic and proteolytic conditions in cells, resulting in a significant degradation of DNA-PK, and it was not even clear if the reported inhibition was for activity or from loss of intact protein. Lastly, we have additional results showing that TLKs and Rad9(S328) phosphorylation are involved in the restart of stalled replication forks after release from hydroxyurea and that TFP greatly extends this process with a delay of its linked phosphorylation of Rad9(S328) after DDR deactivation (Suppl. Figs. S7 and S8). In contrast, DNA-PK is not known to be involved in replication restart and is rather specific for NHEJ. While identifying the molecular target may not seem that important in terms of their potential use as adjuvants in clinical

trials, in reality, the best way to design an effective study is to have available intermediate biomarkers, like P-Rad9, that can also be measured in blood lymphocytes to establish the individual doses. Thus, knowing the mechanism/target of the therapy is important. While we were completing our studies, a paper was published that reported the identification of THD during a screen to find compounds that selectively target cancer stem cells,⁴⁶ therefore adding another potential anticancer target to the use of THD. More specifically, the authors used a presumed distinction between normal pluripotent cells (hPSC) and CSC, based on Oct4-GFP expression, to find compounds that induce differentiation to overcome neoplastic self-renewal. Assuming that this is conceptually applicable to most cancers, many of us think that most cancer cells are “stem cells” by at least the basic definition of unlimited self-renewal; their further conclusions that “these results suggest that dopamine receptors may serve as a biomarker for diverse malignancies” are not subsequently supported in their study. Certainly, we have not found very many cancer cell lines that were sensitive to THD for growth at the concentrations that we used (Suppl. Fig. S5). All those cell lines were derived from patients, and there are very few reports of DR expression in cancer lines, like the small lung cancer cell line NCI-H69. Others have already questioned these conclusions on the mechanistic involvement of the DR as an explanation for the results. For example, why did they not try to knock down DRs in cancer stem cells? Or block DRs with highly specific antibodies? Most tellingly, clozapine, an atypical antipsychotic and DR antagonist (among other activities), also exhibited selective killing of cancer stem cells, but the authors used it at 100 μ M (much greater than its receptor activity range) and only mentioned this important fact in the figure itself. Another recently implicated target of TFP is the FOXO1-KLF6 axis that regulates EGFR signaling.⁴⁷ These authors sought a pharmacologically and clinically viable approach to activate FOXO1 by retaining nuclear localization and overcoming the mislocalization observed in lung adenocarcinoma cell lines and identified TFP in a screen, somewhat analogous to the already mentioned study of Kau and colleagues,²⁸ and unfortunately similarly flawed because they observed a very modest effect on nuclear retention of FOXO1 only at 20 μ M of TFP, which is nonspecifically toxic in most cells that we have studied. Although the authors mention that there was no increase in apoptosis in H1650 cells treated with 20 μ M of TFP, unfortunately, it is reported as data not shown.⁴⁷

Cancer and Schizophrenia. Since these drugs are used for the treatment of severe mental illness, we looked at the literature on the risk of cancer among individuals with schizophrenia. Surprisingly, despite the fact that patients with schizophrenia should have high comorbidity factors that should increase the cancer risk, it is a known paradox in the field that the risk of developing cancer is generally lower

than what might be expected for both male and female common cancers.⁴⁸⁻⁵⁰ Below, we discuss the case of BCA in individuals with schizophrenia in whom more current information from larger studies is available, and we propose a possible explanation for these unexpected epidemiological observations.⁵¹ A recent preclinical trial at Dana-Farber Cancer Institute was designed to establish doses of an oral PARP inhibitor for BCA prevention in BRCA mutation carriers. The underlying hypothesis was that BRCA-deficient tumor cells must rely upon alternative DNA repair pathways to maintain sublethal levels of DNA damage and that PARP inhibitors block these back-up DNA repair pathways, promoting the accumulation of lethal levels of DNA damage but spare normal cells that retain BRCA activity. The epidemiological evidence supports a similar scenario with TFP as an inhibitor of TLKs and hence DNA repair in individuals with schizophrenia, possibly providing similar chemoprevention activity as a PARP inhibitor. An alternative explanation is that elevated TLK1B or TLK2 expression is a critical transition during the development or progression of many BCAs, and pre-emptive inhibition of these kinases by phenothiazines could choke a key step in the formation of breast tumors.

TLKs, Schizophrenia, and BCA. Some phenothiazine antipsychotics (specifically, TFP, THD, PPH, PMZ, TEZ) that have been used to treat patients since the 1950s have been identified as TLK inhibitors. The link between schizophrenia therapy and TLK inhibition is an interesting possibility because if the TLK-inhibiting properties of these drugs are effective against BCA, then one would expect that women who had been prescribed these drugs might display 1) a lower incidence of BCA compared to those who did not take the drugs and/or 2) an improved outcome after BCA diagnosis. While women with schizophrenia have multiple risk factors for BCA that might lead to expectations of a substantially increased risk (smoking, obesity, low exercise, poor diet, treatment-induced hyperprolactinemia), results of a systematic review of such studies¹⁷ revealed that the incidence is decreased, similar, or only slightly increased from that of the general population. Various hypotheses have been proposed for such findings, including protective genetic effects⁵²⁻⁵⁵ and antitumor properties of neuroleptics.⁵⁶⁻⁵⁸

Schizophrenia and CaP. An analysis from 5 independent studies of CaP incidence in individuals with schizophrenia revealed in each case a significant decrease in incidence ranging from 0.49 to 0.76.⁴⁹ Proposed explanations for this were the following: specific genetic factors; antipsychotic drug effects, either by being cancer protective or decreasing testosterone, or both; and lifestyle differences, such as prolonged hospitalization, resulting in a decreased opportunity for heterosexual intercourse. However, the antitumor property of TLK inhibitors was not yet investigated in humans and provides a testable hypothesis for the lack of increased risk for BCA and CaP.

Conclusions. Our research supports a case for the implementation of clinical trials for both CaP and BCA patients in which carefully adjusted doses of phenothiazine inhibitors of TLKs are used as adjuvants for XRT and RMT to produce cancer-targeted therapy with lower toxicity.

Materials and Methods

TLK1 In Vitro Kinase Assays. TLK1 immune complexes were isolated from 200 μ g of protein extracts using antiserum made in our laboratory and Protein A beads (Protein A/G PLUS-Agarose, sc-2003, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Adsorbed proteins were washed twice in kinase assay buffer (KAB) (50 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 2.5 mM EGTA, 1 mM DTT, 1 mM NaF, 0.2 mM sodium *o*-vanadate, 2.5 μ g/mL leupeptin, 2 μ g/mL aprotinin) and incubated for 15 to 30 minutes at 30°C in KAB supplemented with 10 μ M ATP and 10 μ Ci γ -³²P-ATP (>5,000 Ci/mmol) (GE Healthcare, Little Chalfont, UK). The proteins were separated on 10% SDS-PAGE gels, transferred to nitrocellulose before exposure on a PhosphorImager (STORM 860, Molecular Dynamics (GE), Sunnyvale, CA, USA), and then processed for WB with TLK1 antiserum.

For kinase assays with recombinant TLK1B protein, the reactions were similar and carried out with 10 ng of TLK1B and 50 ng of Rad9 peptide substrate. These were carried out to confirm the initial “hits” from the screen and furthermore to obtain accurate ID50 values for the various inhibitors.

Plasmid In Vitro Repair Reactions. Preparation of nuclear extracts and plasmid religation coupled to chromatin assembly have been described.⁵ Ligation of a blunt end generated by EcoRV and one generated by EcoRI can only be repaired by the generation of a flush end at the EcoRI side, which could either take place by fill-in of the EcoRI overhang and resection of the remaining 5'-ApA overhang or by fill-in with residual dTTP present in the extract. Where indicated, recombinant hRad9 was added at 100 nM of the final concentration as previously described.⁵

QM-Molecular Mechanics (QM-MM) Calculations. The various phenothiazine drugs were initially minimized with the MMFF force field in Discovery Studio 3.1 (Accelrys, San Diego, CA). Geometry optimization and molecular orbital visualization were then achieved using QM-MM methods. Molecules were typed with the CHARM force field with MMFF94 partial charge settings. Density functional theory methods with the VWN-BP gradient-corrected functional were used to calculate QM properties of the molecules. The DN DMol³ basis set was used with self-consistent field convergence at 1×10^{-4} . The frontier orbitals, HOMO and LUMO, were then visualized to compare the active versus inactive drugs.

Animal Studies. All animals used in this study received humane care based on guidelines set by the American

Veterinary Association as well as in accordance with the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research). The experimental protocols involving live animals were reviewed and approved by the Institutional Animal Care and Use Committee of Louisiana State University Health Sciences Center Shreveport. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques. Male and female SCID/bg mice were used, depending on the cell line model.

IHC of Tumors. Sectioning and processing of the tissues were carried out in the Molecular Core Facility (MCF) IHC core of the Department of Cellular Biology and Anatomy with the expert work of Jennifer R. Gill, using automated processes and equipment to provide uniform and standardized results. Indirect labeling was with Elite ABC (RTU Vectastain Elite Reagent, #PK-7100, Vector Laboratories, Burlingame, CA) and DAB (ImmPact DAB, #SK-4105, Vector Laboratories). Light counterstaining was with hematoxylin. Primary antibodies were phospho-Rad9(S328) (cat. #AP3225a, Abgent, San Diego, CA), RPA-70 (cat. #AJ1696a, Abgent), anti-phospho-histone H2A.X (Ser139) (cat. #05-636, Millipore, Billerica, MA), and anti-PCNA (cat. #MAB424, Millipore) and typically used at a 1:400 dilution.

Declaration of Conflicting Interests

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