The novel chemical entity YTR107 inhibits recruitment of nucleophosmin to sites of DNA damage, suppressing repair of DNA double strand breaks, and enhancing radiosensitization.

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Abstract:

**Purpose:** Radiation therapy continues to be an important therapeutic strategy for providing definitive local/regional control of human cancer. However, oncogenes that harbor driver mutations and/or amplifications can compromise therapeutic efficacy. Thus, there is a need for novel approaches that enhance the DNA damage produced by ionizing radiation.

**Experimental Design:** A forward chemical genetic approach coupled with cell-based phenotypic screening of several tumor cell lines was used to identify a novel chemical entity (NCE) that functioned as a radiation sensitizer. Proteomics, comet assays, confocal microscopy and immunoblotting were used to identify the biological target.

**Results:** The screening process identified a 5-((N-benzyl-1H-indol-3-yl)methylene)pyrimidine-2,4,6(1H,3H,5H)trione as a NCE that radiosensitized cancer cells expressing amplified and/or mutated RAS, ErbB, PIK3CA, and/or BRAF oncogenes. Affinity-based solid phase resin capture followed by LC/MS/MS analysis identified the chaperone nucleophosmin as the NCE target. SiRNA suppression of nucleophosmin abrogated radiosensitization by the NCE. Confocal microscopy demonstrated that the NCE inhibited nucleophosmin shuttling to radiation-induced DNA damage repair foci and analysis of comet assays indicated a diminished rate of DNA double strand break repair.

**Conclusion:** These data support the hypothesis that inhibition of DNA repair due to inhibition of nucleophosmin shuttling increases the efficacy of DNA damaging therapeutic strategies.
Translational Relevance

Ionizing radiation is an important tool for the treatment of human cancer and it has been hypothesized that its effectiveness is a consequence of its ability to overload a cancer cell’s ability to respond to DNA damage (1). Emerging research has implicated the contribution of oncogenic driver mutations to radiation resistance. We hypothesized that the DNA damage stress overload paradigm could be exploited in cancer cells for the purpose of radiosensitization. A forward chemical genetics screen identified a novel chemical entity (NCE) that potentiated radiation sensitivity, as assayed in preclinical models. Radiosensitization was a consequence of increased DNA double strand break formation and repair inhibition. The NCE was used as a tool for target identification. This approach identified the chaperone nucleophosmin, a protein known to be required for DNA double strand break repair. Confocal microscopy demonstrated that the NCE prevented nucleophosmin shuttling to sites of DNA double strand breaks. These data identify the nucleophosmin shuttling pathway as a potential target for enhancing the efficacy of DNA damaging therapeutic strategies.
Introduction:

Cytotoxic therapy continues to be a very important tool for the treatment of human cancer. Ionizing radiation is an example of a cytotoxic agent that has a central role in cancer therapy and is used to provide local/regional control of prostate, pancreatic, head and neck, breast, brain, colorectal, lung, metastatic bone, Hodgkin's, ovarian, and uterine cancers. (2).

DNA damage stress overload is a concept that describes the ability of a therapeutic regime to overwhelm the DNA damage response pathways in a cancer cell (1). This concept helps explain the efficacy of ionizing radiation, which produces DNA double strand breaks, single strand breaks, and base modifications (3) due to generation of oxygen radicals. The predominant radical yield following x- or γ-irradiation consists of hydroxyl and superoxide anion radicals (discussed in (4)) and it is the hydroxyl radical which generates the formation of toxic DNA double strand breaks.

Emerging research has shown that cancer driver mutations can impact a cell's response to radiation-induced DNA damage (5, 6). Driver mutations are defined as mutations that initiate and sustain tumor progression (1). Tumors may express several driver mutations (7) and each can independently affect compensatory DNA damage response pathways. Colorectal cancer provides an excellent example. A colorectal tumor may harbor a gain of function KRAS mutation as well as overexpress EGFR (8). Each of these mutations can independently confer a radiation resistance phenotype (9, 10) and negate therapeutic DNA damage stress overload strategies (1, 11). Thus, there is a need for novel approaches that enhance the DNA damage produced by cytotoxic agents.

We used a forward chemical genetic approach integrated with cell-based functional screening (12) to test the hypothesis that components of the DNA damage response pathway can be exploited for the purpose of radiosensitizing tumor cells expressing amplified and/or mutated oncogenes. A novel chemical library was synthesized and cell-based phenotypic assays were employed (13) to screen for sensitivity to ionizing radiation. The screening process identified a 5-((N-benzyl-1H-indol-3-yl)methylene)pyrimidine-2,4,6(1H,3H,5H)trione, denoted as YTR107, as a...
compound that increased the number of DNA double strand breaks formed per Gy and slowed the repair of DNA double strand breaks.

Affinity-based solid-phase resin capture was then employed for identifying potential biological targets. YTR107 linked to a solid-phase resin was used to capture cell lysate. LC/MS/MS analysis of the captured protein identified the chaperone nucleophosmin (NPM) in the complex of proteins that bound to the affinity resin. Recombinant NPM was used to demonstrate that YTR107 can directly bind to NPM. NPM is a molecular chaperone involved in anaplastic large cell lymphoma (14), as well as in cellular processes such as centrosome duplication, ribosome biogenesis, cell cycle progression (15), and repair of DNA damage (16). T199 phosphorylated (pT199) NPM shuttles to sites of DNA double strand breaks in a RNF8 ubiquitin-dependent manner (16). Failure of pT199-NPM to localize to sites of DNA double strand breaks results in inhibition of their repair (16). We found that YTR107 inhibited NPM shuttling to ionizing radiation-induced DNA damage repair foci, as marked by γH2AX. Taken all together these results support the hypothesis that YTR107 inhibits DNA repair by inhibiting NPM shuttling and thus increases the efficacy of DNA damaging therapeutic strategies.
Materials and Methods:

Cell lines and reagents: Microplasma-free human HeLa cervical adenocarcinoma cells, HT29 colorectal adenocarcinoma cells, Panc-1 exocrine pancreatic cancer cells, MDA-MB-231 mammary adenocarcinoma cells, HCC1806 breast adenocarcinoma cells, H460 non-small cell lung cancer (NSCLC) cells, and human D54 glioblastoma cells were grown in their recommended media. HT29 cells were grown as xenografts as described in (17). NSC348884 was obtained from the National Cancer Institute, NIH.

The following antibodies were used: RPA2 (Abcam), Chk1 (Santa Cruz Biotechnology), pS317 Chk1 (Cell Signaling Technology), pT199 NPM (Abcam), NPM (Invitrogen), and pS139 H2AX (Millipore). Carboplatin was purchased from Sigma-Aldrich.

Colony formation assay: Plating efficiency and colony formation were quantified as described in (18).

Irradiation: Cells were inoculated into 100 mm Petri dishes and irradiated with a Mark 1 $^{137}$Cs irradiator (2.0 Gy per min). A Pantek 300 kVp/10 mA X-ray machine (2.1 Gy per min) was used to irradiate xenograft tumors.

Synthetic chemistry: A series of (Z)-5-((N-benzyl-1H-indol-3-yl)methylene) imidazolidine-2,4-dione and 5-((N-benzyl-1H-indol-3-yl)methylene)-pyrimidine-2,4,6(1H,3H,5H)-trione derivatives that incorporate a variety of substituents in both the indole and N-benzyl moieties were synthesized, as described in (13). Twenty two compounds were synthesized and structurally characterized by $^1$H and $^{13}$C nuclear magnetic resonance spectroscopy, gas chromatography-mass spectroscopy, and elemental combustion analysis (13).
**Comet assay:** The Neutral Comet Assay was performed using the Comet Assay kit from Trevigen following the manufacturer’s instructions. Comets were analyzed using CometScore software.

**PARP activity:** PARP activity was measured using a kit from Trevigen following the manufacturer’s instructions.

**Immunofluorescence:** Lab-Tek II chamber slides (Nalge Nunc International) were used as a platform for cell growth. HeLa cells were grown to 50% confluency and treated with YTR107 (50 µM) for 30 minutes before exposure to 4 Gy radiation. Cells were then allowed to grow for 1.5 hours at 37°C before fixing with cold 4% paraformaldehyde at 4°C for 20 min. Dimethyl sulfoxide (DMSO) was used as solvent control. Paraformaldehyde-fixed cells were permeabilized by treatment with 0.1% Triton X-100 in phosphate-buffered saline (PBS). Cells were then blocked in 3% bovine serum albumin in PBS for 1 hour at 25°C. pNPM rabbit monoclonal (Abcam) and γ-H2AX mouse polyclonal antibodies (Millipore) were then applied at 1:200 dilutions in 1x BSA overnight at 4°C and subsequently labeled with Alexa647 or Alexa486 fluorescent dye-conjugated secondary antibodies, respectively. DAPI (Invitrogen) was used as the nuclear stain. Images were acquired using an Olympus FV 1000 inverted laser scanning confocal microscope.

**Quantification of colocalized protein:** Colocalization of pT199 NPM and γ-H2AX was quantified using the “Colocalization” module of Metamorph software. Colocalization data was obtained from 84 cells per condition and represents two independent experiments.

**Tumor growth inhibition:** These studies were approved by the Vanderbilt Institutional Animal Care and Use Committee and performed under guidelines outlined in The Guide for the Care and Use of Laboratory Animals. Hindlimbs of homozygous nu/nu athymic nude mice (Charles River Laboratories), ~6–8 wk of age, were subcutaneously implanted with HT29 human colorectal cancer
cells. When tumors achieved a size of approximately 180 mm³ (calculated 3 times per week according to the formula: length x (width)²/2) they were randomized to the following protocols: 7 daily i.p. injections of (a) DMSO (25 μl) or (b) 10 mg/kg of YTR107 in DMSO (25 μl) followed 30 min later by (c) 0 Gy or (d) 3 Gy of x-rays (300 kVp/10 mA). Six mice were assigned to each protocol. Mice were shielded such that only the tumors were irradiated. Digital calipers were used to obtain the length and width of each tumor. The starting volume of each mouse was normalized to 1.0.

Proteomics: YTR107 and benzoic acid (control) were covalently linked to Dynabeads M-270 Amine (Invitrogen, CA) in separate reactions. The structure of YTR107 containing a linker moiety is shown in Supplemental Figure 4 and denoted as YTR119. Total protein lysate was prepared from HT29 cells in extraction buffer (1 mM CaCl₂, 150 mM NaCl, 10 mM Tris (pH 7.4), 1% Triton X-100, and protease inhibitor cocktail (Sigma). An equal amount of protein lysate was added to the magnetic beads and incubated for 3 hr at 4°C with mixing. Unbound proteins were removed using magnetic separation; the beads were washed with TBS-T (20 mM Tris, 150 mM NaCl, 0.1% Triton X-100) four times. Bound proteins were eluted with EB (20 mM Tris, pH 8.0, 2M NaCl). Proteins were resolved by SDS-PAGE and stained with colloidal coomassie blue stain (Invitrogen). Those proteins that appeared by colloidal coomassie blue staining to be specific for YTR107 were excised for identification by mass spectrometry. These bands were subjected to in-gel tryptic digestion and the resulting peptides analyzed by high performance liquid chromatography coupled tandem mass spectrometry (LC-MS/MS). Briefly, peptides were resolved using an Eksigent 1D+ HPLC equipped with an AS1 autosampler on an 18 cm Jupiter (3 micron, 300A) 100 um internal diameter, self-packed analytical column coupled directly to an LTQ (ThermoFisher) via a nanoelectrospray source. A full scan mass spectrum followed by 5 data-dependent tandem mass spectra (MS/MS) were collected throughout the run using dynamic exclusion to minimize acquisition of redundant spectra. MS/MS spectra were searched against a human protein database (UniprotKB v155) using

Presence of NPM the pulled down protein complex: Biotinylated YTR107 and biotinylated benzoic acid (PNR-4-31; structures shown in Supplemental Figure 4) were synthesized and allowed to bind streptavidin magnetic beads (Promega) in 50% DMSO in PBS. Unbound compounds were removed by washing with 50% DMSO and PBS. HT29 cell extract was prepared in RIPA buffer (10 mM Tris, pH7.2, 150 mM NaCl, 1% deoxycholic acid, 5 mM EDTA, 1% Triton X-100, 0.1% SDS). Proteins were allowed to bind to the magnetic beads for 1 hr and washed 5 times with RIPA buffer and 3 times with RIPA buffer containing 500 mM NaCl. The beads were heated with 15 μl of 5x Laemmli buffer and resolved on SDS-PAGE and western blotted for NPM.

Recombinant NPM: Recombinant His-NPM was expressed from a pET28a vector, a gracious gift from Dr, Jason Weber, Washington University) and purified using Ni-NTA agarose gel. 10 ug of NPM was added to biotinylated-YTR107 or benzoic acid (control) bound to streptavidin magnetic beads as described above and incubated for 30 min while mixing at 4°C. The magnetic beads were washed 5 times to remove unbound protein, boiled with 5x Laemmle buffer and immunoblotted for NPM.

Fractionating soluble and chromatin bound nuclear protein: Preparation of nuclear extracts was performed as described by Groisman et al (19). In brief: cells in hypotonic buffer (10 mM Tris-HCl, pH 7.3, 10 mM KCl, 1.5 mM MgCl$_2$, 10 mM β-mercaptoethanol, and protease inhibitor cocktail) underwent Dounce homogenization. Nuclei were collected by centrifugation and resuspended in extraction buffer (15 mM Tris-HCl, pH 7.3, 1 mM EDTA, 0.4 M NaCl, 1 mM MgCl$_2$, 10% glycerol, 10 mM β-mercaptoethanol, and protease inhibitor cocktail). After a 30 min/4°C incubation, nuclei
were centrifuged at 20,000 × g for 30 min (4°C). The supernatant was washed and subjected to immunoblotting.

Results:

**Cell-based phenotypic screening:** The initial screening was performed using human HT29 colorectal adenocarcinoma cells that express mutated p53, activated PIK3CA, and BRAF<sup>V600E</sup> (Supplemental Table 1). The cells also express amplified ErbB1 and wildtype HRAS. All screening was performed using compound concentrations that did not reduce plating efficiencies below 70% following a 2 hr/37°C exposure. Radiosensitization was quantified from colony formation assays. Colony formation provides a rigorous test of cell viability, cell cycle proliferation, and DNA damage sensitization by requiring individual cells to divide and proliferate through their cell cycle a minimum of 7 times after sustaining DNA damage (18).

Only 3 of the 22 compounds tested yielded statistically significant radiosensitization of cells exposed to 4 Gy (13). Compound YTR107 was 2 fold more effective than the other two radiosensitizing compounds (p < 0.05 Student's t test) and therefore was chosen for further investigation (structure shown in Figure 1). The dose response curves and dose modifying factors shown in Figure 1 indicate that exposure to YTR107 significantly increased the radiation sensitivity of HT29 colorectal adenocarcinoma, D54 glioblastoma, PANC1 pancreatic carcinoma, MDA-MB-231 breast adenocarcinoma cells, and H460 NSCLC cells. Supplemental Table 1 lists known mutations present in these cancer cells. The dose modifying factor (DMF) shown in Figure 1 is defined as the dose of radiation required to reduce survival to 10% in YTR107 treated cells divided by the dose of radiation required to reduce survival to 10% in cells treated with vehicle control.

An alternative method of comparing effectiveness is to compare survival produced by the clinically significant dose of 2 Gy. In HT29 cells, exposure to 2 Gy decreased survival to 0.79 +/- 0.04 (SEM). Exposure to 25 μM YTR107 plus 2 Gy reduced survival to 0.39 +/- 0.04 (SEM), p <
0.0001, Student's t test. Similar results were obtained in D54, PANC1, MDA-MB-231, and H460 cells when exposed to 25 μM YTR107 plus 2 Gy (p ≤ 0.01, Student's t test).

YTR107 potentiates radiation-induced growth delay of HT29 tumor xenografts. HT29 tumor bearing mice were administered 7 daily fractions of the following treatments: 1) i.p. injection of solvent control DMSO; 2) i.p. injection of 10 mg/kg of YTR107 (q.d.x7); 3) i.p. injection of solvent control followed 30 min later with 3 Gy (q.d.x7); 4) i.p. injection of 10 mg/kg of YTR107 followed 30 min later by 3 Gy (q.d.x7). The fold increase in tumor volume is shown in Figure 2. In untreated mice tumor volume increased 4 fold in 5 days. In mice administered YTR alone, tumor volume increased 4 fold in 6 days. In mice administered 7 daily 3 Gy fractions, tumor volume increased 4 fold in 7 days. However, in mice administered 7 daily fractions consisting of YTR107 followed by 3 Gy, it took 32 days for tumor volume to increase 4 fold. An alternative method of comparison was also used. Twelve days after irradiation (19th day of experiment, Figure 2), tumor volume increased 7.7-fold +/- 1.0 (SEM) for irradiation alone vs 1.8 fold +/- 0.24 (SEM) for tumors administered YTR107 plus irradiation (p = 0.001, Student's t test).

It is important to reiterate that radiosensitization by this NCE occurred at concentrations that did not lower the plating efficiency of the cell based assays below 70%, compared to solvent control. Furthermore, YTR107 was well tolerated in mice. Five female C57BL/6J mice were administered 10 mg/kg i.p. YTR107 for 5 consecutive days and 5 mice were administered solvent alone. Thirty five days after injection mice were euthanized and subjected to necropsy by a veterinarian trained in veterinary pathology. Gross and histological examination of liver, lung, thymus, heart spleen, cerebellum, pancreas, small intestine, kidney, and adrenal gland did not reveal evidence of toxicity that could be attributed to injection of YTR107 (data not shown). Both a complete blood count and a white blood cell differential count were performed. No significant differences were noted (Supplemental Table 2). The weights of the mice measured over the 35 day interval are shown in Supplemental Figure 1. There was not a statistically significant difference in
weights of mice administered the two treatments (p> 0.05 Student’s t test). We interpret these data to indicate that the change in radiosensitization cannot be considered a secondary consequence of toxicity.

Replication stress: The data shown in Figure 3A demonstrate that incorporation of $^3$H thymidine into TCA precipitatable DNA is inhibited in cells exposed to YTR107 for 2 hrs at 37°C (p < 0.0001, ANOVA). Inhibition of DNA synthesis can be accompanied by stalled DNA replication forks. Stalled replication forks result in accumulation of single stranded regions. RPA binds to single stranded DNA and immunofluorescent imaging can be used to detect the presence of such regions (20). HeLa cells have excellent optical properties for immunofluorescent imaging and therefore were used to measure binding of RPA2 to single stranded DNA following a 2hr/25μM exposure to YTR107. Representative images are shown in Figure 3B. Approximately 45% of control cells exhibited RPA immunofluorescent images that are characteristic of stage IB S phase cells in which discrete replication foci have merged together (21). In contrast, approximately 65% of YTR treated cells were immunofluorescence-positive (Figure 3B). Given that DNA synthesis is inhibited in YTR107 treated cells, we interpreted the increase in RPA immunofluorescence to be a consequence of increased regions of single stranded DNA. RPA2 binding to single stranded DNA results in recruitment of ATR (22) and subsequent ATR-mediated phosphorylation of Chk1 kinase at Ser residues 317 and 345 (23). As shown in Figure 3C, exposure to YTR107 produced a robust phosphorylation at Ser 317 of Chk1.

Histone H2AX is phosphorylated at Ser139 in response to DNA double strand breaks. Histone H2AX is also phosphorylated in response to single stranded DNA regions formed as a result of replication fork stalling ((24) and references therein). Exposure to 25 μM YTR107 for 2hrs resulted in H2AX phosphorylation in HT29 cells (Figure 3D). Similar results were observed in MCF-7 cells (data not shown).

Chk1 signaling is essential for implementing the G2/M checkpoint (25). Consistent with the Chk1 and H2AX phosphorylation, cells accumulated in G2/M following exposure to YTR107.
(2hrs/25 μM) (Supplemental Figure 2). Taken all together, the data presented in Figure 3 and Supplemental Figure 2 demonstrate that exposure to YTR107 is accompanied by inhibition of DNA synthesis, RPA binding to single stranded DNA, ATR-mediated Chk1 phosphorylation, phosphorylation of H2AX, and activation of the G2/M check point. These events are the hallmark of an activated replication stress sensor-response pathway that is responding to stalled replication forks, as described by Branzei and Foiani (26).

We next determined if YTR107 inhibited poly(ADP-ribose) polymerase (PARP) activity. This was accomplished by measuring the ability of cell lysate obtained from cells exposed to 25 or 50 μM YTR107 (2hr/37°C) to catalyze the incorporation of biotinylated PARP into histone proteins in a 96-well format. We found that PARP activity derived from 50 μg cellular protein was independent of the YTR107 exposure (Supplemental Figure 3).

Identification of NPM: In order to identify potential targets, both YTR107 and benzene (negative control) were linked to sepharose beads and allowed to bind total HT29 protein lysate. The structure of the linked YTR107 molecule is shown in Supplemental Figure 4. Bound protein was stringently washed and eluted. Colloidal Comassie Blue staining of SDS-PAGE separated protein revealed the presence of YTR107-specific peptide bands that were excised, subjected to tryptic digestion, and analyzed by LS-MS-MS. The analysis revealed that the molecular chaperone NPM was one of the proteins captured by YTR107 immobilized to sepharose beads (53.4% sequence coverage; see Supplemental Figure 5). The majority of the remaining proteins captured by the YTR107 resin were identified as proteins that can bind to NPM.

To confirm that YTR107 was binding to NPM, cellular protein lysate was added to biotinylated YTR107 or biotinylated benzoic acid (PNR-4-31) affinity beads (See Supplemental Figure 4 for structures). Captured protein was washed, eluted, and subjected to immunoblotting with antibody to NPM. The immunoblot shown in Supplemental Figure 6 illustrates that YTR107 affinity beads were able to trap NPM whereas biotinylated benzoic acid was not. The experiment
was then repeated using purified recombinant NPM. Biotinylated YTR107, but not biotinylated benzoic acid, was able to capture recombinant NPM (Supplemental Figure 6).

NSC348884 is a small molecule shown to disrupt NPM oligomerization and function (27). Exposure of HT29 cells to 10 μM NSC348884 for 30 min prior to, during, and for 90 min after irradiation produced a statistically significant degree of radiosensitization (Supplemental Figure 7A; p < 0.04, Student’s t test). In addition, siRNA targeting of NPM (SMARTpool from Dharmacon) was used to suppress NPM expression in HeLa cells. Control cells were transfected with a non-targeting siRNA. Cells were exposed to YTR107 and irradiated (Supplemental Figure 7B). In cells transfected with control siRNA, YTR107 produced a statistically significant degree of radiosensitization (p < 0.0001). However, YTR107 failed to radiosensitize cells transfected with NPM siRNA (p > 0.05 Student’s t test).

Inhibition of NPM shuttling to sites of DNA double strand breaks in YTR107 treated cells. pT199-NPM has been shown to participate in repair of radiation-induced double strand breaks (16). Specifically, nucleoplasm soluble pT199-NPM translocates into an insoluble chromatin fraction as it associates with BRCA1-BARD1 complexes at sites of DNA double strand breaks. We monitored the solubility of pT199-NPM in irradiated cells using the biochemical fractionation technique described by Groisman et al. (19). Congruent with the results obtained by Koike et al. (16), pT199-NPM solubility decreased in irradiated cells. pT199-NPM solubility decreased by 50%, 90 min after administering 4 Gy and by 60% following administration of 6 Gy (Figure 4A). However, exposure to YTR107 prior to and after irradiation inhibited pT199 NPM redistribution.

Indirect immunofluorescent imaging by confocal microscopy was used to determine if YTR107 affected NPM/γH2AX colocalization (Figure 4B). HeLa cells were grown to 50% confluence, treated with YTR107 (50 μM) for 30 min before exposure to 4 Gy, allowed to recover for 90 min at 37°C, and then analyzed by confocal microscopy. While barely detectable in unirradiated cells, γH2AX (labeled with Alexa 488 (Green)) was clearly detected 90 min after irradiation and
exhibited a typical punctate pattern (Figure 4B). Consistent with our previous observations, we saw some increase in γH2AX immunofluorescence in cells that were treated with YTR107 alone without irradiation (Figure 4B). pT199-NPM (labeled with Alexa 647 (red)) was detected in both normal and irradiated conditions (Figure 4B).

Exposure to 4 Gy induced pT199 NPM colocalization with γH2AX (yellow color in the merged image, Figure 4B). However, treatment with YTR107 markedly decreased this colocalization, as quantified using Metamorph software. The mean area of overlap between red pNPM signals and green γH2AX signals is a measure of colocalization and was calculated for cells exposed to 4 Gy with or without treatment with YTR107 (Figure 4C). Data are expressed in arbitrary units (means ± SD) as the mean area overlap of red and green channels from 84 cells per condition. The data indicated a decrease in colocalization signal between pNPM and γH2AX in cells that were treated with YTR107 and irradiation (p value <0.0001, Student's t-test).

DNA double strand break repair: The repair of DNA double strand break (DSB) induced by ionizing radiation is biphasic. Sixty percent of DSBs are repaired rapidly, with a rejoining half-life on the order of minutes (reviewed in (28)). The remaining DSBs are considered ‘persistent’ and are repaired slowly, exhibiting rejoining half-lives on the order of hours.

Phosphorylation of histone H2AX at Ser139 represents one of the earliest events following formation of DNA DSBs. Careful comparisons of γH2AX elimination kinetics with the kinetics of DNA DSB rejoining have demonstrated a direct correlation between the number and repair of the ‘persistent’ DNA double strand breaks and the formation and elimination of γH2AX (28).

We quantified γH2AX immunofluorescence 90 min after irradiation of HT29, PANC1, and H460 cells by flow cytometry. We found that γH2AX immunofluorescence was significantly elevated in irradiated HT29 and PANC1 cells exposed to YTR107 compared to cells irradiated in DMSO (solvent control), Supplemental Table 3. NSCLC H460 cells were irradiated in the presence of either DMSO or YTR107 and then incubated at 37°C for 1.5 or 2.5 hrs prior to quantifying γH2AX...
immunofluoresence. Exposure to YTR107 slowed the elimination of γH2AX immunofluoresence, a surrogate for repair of DNA DSBs, Supplemental Table 4.

A neutral comet assay (29) was also used to assess formation and repair of DNA double strand breaks following x-irradiation. HT29 cells were incubated at 37°C for 2 hrs in the absence or presence of YTR107 (25μM), irradiated at 4°C, and then immediately processed for the comet assay (Figure 5 A-D). DNA double strand breaks were not detected in cells exposed to YTR107 alone (Figure 5B). However, the comet heads from YTR107 treated cells did not yield sharp images (compare DMSO control, Figure 5A to 5B). This may be a consequence of YTR107-mediated replication stress that induced single strand breaks. Exposure of cells to YTR107 prior to and during irradiation at 4°C, a temperature that inhibits repair, significantly increased comet tail length (compare Figure 5C to 5D). These data indicate that formation of radiation-induced double strand breaks was potentiated by YTR107.

HCC1806 breast adenocarcinoma cells are estrogen receptor negative, progesterone receptor negative, p53 null, and over express HER2/neu (30). The HCC1806 cells are radiosensitized by exposure to 25 μM of YTR107 (Figure 5E). The repair of DNA double strand breaks was quantified in these cells by measuring comet moment (Figure 5F; defined in (29)). Cells were exposed to 25 μM YTR107 for 30 min at 37°C, irradiated at 4°C, and immediately processed (at time zero: no repair) or allowed to repair at 37°C. These data demonstrate that in irradiated cells, YTR107 increased the formation of DNA double strand breaks and slowed their repair.
Discussion: The challenge to radiation oncology is to provide definitive local/regional control of tumors harboring complex genetic profiles. Comprehensive sequencing of cancer cell genomes has revealed a diversity of disease-specific mutations. Examples include MLL2 mutations in medulloblastoma and IDH1 mutations in glioblastoma (31, 32). Additionally, the sequencing data has shown the presence of driver mutations that populate well characterized signaling pathways that have established roles in oncogenesis and are common to many types of cancer (31). A collateral consequence of such oncogenic mutations is context-dependent resistance to therapeutic regimens.

We hypothesized that DNA damage response pathways harbor biological targets that could be exploited for the purpose of producing context-independent sensitization. To test this hypothesis we used a forward chemical genetics approach that employed cell-based phenotypic screening of cancer cells for the purpose of identifying novel chemical entities (NCE) that could enhance therapeutic effectiveness. The screen identified YTR107, a 5-((N-benzyl-1H-indol-3-yl)methylene)pyrimidine-2,4,6(1H,3H,5H)trione as a compound that potentiated the radiation sensitivity of several diverse cancer cell lines and a HT29 xenograft.

YTR107 was used as a tool for identifying the biological target responsible for sensitization. One candidate protein identified was NPM. NPM is considered a chaperone that shuttles between various cellular compartments. Originally it was described as a nucleoli protein involved in ribosome biogenesis (33). Recent research has shown that it is involved in many aspects of cell physiology (33). Emerging research has also shown that NPM participates in DNA damage responses. NPM is essential for embryonic development and maintenance of genomic stability (34, 35). Loss of NPM can induce replication stress. NPM co-purifies with the DNA polymerase α/primase complex (36). Using purified and recombinant NPM in a cell free system, Takemura et al. (36) have shown that NPM binds to and stimulates the activity of DNA polymerase α by as much as 3 fold. Thus, loss of NPM would be predicted to diminish DNA synthesis. Consistent with that hypothesis, Colombo et al (34) found that RNAi-mediated suppression of NPM in MEFs inhibited...
incorporation of BrdU into DNA and induced cell cycle arrest. Immunoblotting of NPM−/− embryo lysates indicated enhanced H2AX phosphorylation at Ser139 compared to lysates from wild type embryos (34). The results reported in these studies provided a rationale for focusing on the role of NPM in YTR107-mediated radiosensitization.

Recently Koike et al., (16) found that pT199 NPM recruitment to sites of DNA double strand breaks is required for DNA repair. Recruitment relies upon the NPM ubiquitin binding motif-like domain interacting with an unknown substrate polyubiquitinated by RNF8 (16). RNF8 is recruited to sites of DNA damage via interaction with MDC1, allowing recruitment of BRCA1-Abraxas-RAP80 complexes. This event is followed by recruitment of BRCA2-RAD51 complexes, thus licensing checkpoint signaling and homologous end joining (37). This DNA damage response pathway is initiated by both single strand DNA regions produced by replication fork stalling and DNA double strand breaks. Failure to recruit NPM to this repair pathway stymies repair (16).

Use of an affinity-based solid phase resin followed by LC/MS/MS or immunoblotting of captured protein demonstrated the presence of NPM in the protein complex bound to YTR107. Confocal microscopy demonstrated that pT199 nucleophosmin relocated to sites of DNA double strand breaks, denoted by γH2AX foci. We found that exposure to YTR107 prevented colocalization. Koike et al., (16) has shown that repair was inhibited when pT199-NPM failed to localize to sites of DNA double strand breaks. Similarly, radiation-induced double strand break repair was inhibited in cells exposed to YTR107, coincident with failure of pT199-NPM to colocalize with γH2AX foci.

The increase in radiation sensitization, measured using a colony formation assay, which requires cells to undergo a minimum of 7 cell divisions, is consistent with an inhibition of DNA double strand break repair. Although Koike et al. (16) did not observe a correlation between the 8 fold inhibition of DNA double strand break repair and cell viability, this may be a consequence of the use of a colorimetric assay that measured viability 4 days after irradiation. Irradiated cancer cells can undergo up to 7 cell divisions prior to senescence, apoptosis, necrosis, or mitotic catastrophe.
Viability measurements made just 4 days after irradiation may not capture the full expression of cell death.

We also found that YTR107 produced a damage response similar to that produced by replication fork stalling: RPA coating of single strand DNA, Chk1 phosphorylation by activated ATR, phosphorylation of the histone H2AX, followed by the activation of the G2 check point (20-23). The observation that YTR107 increased the initial level of DNA double strand breaks is interpreted to be a consequence of the interaction of the DNA single strand breaks formed by replication stress with those generated by x-irradiation. Currently, it is not known if YTR107-mediated ‘fork collapse’ is a consequence of deregulated NPM. NPM regulates DNA polymerase α activity (36), which may be disrupted by YTR107. In addition, NPM is a well characterized histone chaperone for the H2A/H2B histone dimer (reviewed in (40)) and histone organization requires chaperoning during DNA replication. Failure to correctly disassemble and reassemble histones during replication could be expected to induce a replication stress. Nucleosomes are also disrupted during the repair of DNA double strand breaks (discussed in (41)). Thus, one may hypothesize that YTR107-mediated disruption of NPM-mediated histone chaperone activity results in replication fork stalling and inhibition of DNA strand break repair. Alternatively, YTR107-mediated replication fork stress may be independent of NPM since Koike et al (16) did not report replication fork collapse in cells expressing T199A-NPM. Future research will determine the answer to this question.

In summary, these data support the hypothesis that YTR107 inhibits the repair of DNA double strand breaks by deregulation of NPM shuttling and identify this pathway as a potential target for enhancing the efficacy of DNA damaging therapeutic strategies in cells harboring RAS, BRAF, ErbB, and/or PIK3CA driver mutations/amplifications.
References


Figure Legends:

Figure 1: Radiation sensitivity is enhanced by exposure to YTR107. The Figure illustrates the chemical structure of YTR107 and the dose response curves of D54, PANC1, HT29, MDA-MB-231, and H460 cells following x-irradiation. Cells were exposed to the indicated concentrations of YTR107 at 37°C for 30 min prior to, during and after 1.5 hrs after irradiation. The dose modifying factor (DMF) is defined as the dose of radiation required to reduce survival to 10% in YTR107 treated cells divided by the dose of radiation required to reduce survival to 10% in cells treated with vehicle control.

Figure 2: Tumor growth delay following treatment with YTR107 plus irradiation. Athymic nu/nu mice with HT29 hindlimb tumors received daily i.p. injections of DMSO or 10 mg/kg of YTR107 for 7 consecutive days, followed 30 min later by administration of 0 or 3 Gy.

Figure 3: YTR107 induces replication stress in HT29 cells. A. Cells were exposed to the indicated concentrations of YTR107 for 2 hrs at 37°C. Cells were labeled with 8 μCi of [3H] thymidine, washed and DNA precipitated with ice cold 10% trichloroacetic acid. Precipitated DNA was solubilized in 0.2N NaOH. [3H] Thymidine incorporation was quantitated by scintillation counting; HT29 cells were exposed to 25 μM YTR107 for 2 hr at 37°C and then analyzed for RPA2 immunofluorescence and DAPI counterstaining (Panel B), or immunoblotted for pS317Chk1 and total Chk1 (panel C) or γH2AX and GAPDH (Panel D).

Figure 4: YTR107 inhibits pT199 nucleophosmin translocation to sites of DNA damage. A. Nuclear extracts were prepared as described in Materials and Methods. Nonchromatin bound protein immunoblotted for the presence of pT199 nucleophosmin; B Confocal images of cells stained for
pT199 NPM and γH2AX; C. Quantification of pNPM and γH2AX colocalization, as described in the text.

Figure 5: YTR107 inhibits repair of DNA double strand breaks. Cells were exposed to DMSO (Panel A) or YTR107 (25 μM; Panel B) for 2hrs at 37°C and then administered 0 (Panels A and B) or 3 Gy (Panels C and D) at 4°C. Immediately after irradiation cells were subjected to a neutral comet assay; Panel E illustrates radiosensitization of HCC1809 cells by 25 μM YTR107. Cells were exposed to YTR107 for 30 min prior to, during and for 90 min after 4 Gy. Panel F illustrates the formation and repair of DNA double strand breaks of cells exposed to either DMSO or YTR107 for 30 min at 37°C, irradiated at 4°C, and allowed to repair at 37°C for up to 150 min.
Figure 2

HT29 Xenograft

Tumor Volume Fold Increase

Days

DMSO

YTR107

3 Gy

YTR107 + 3 Gy

10 mg/kg YTR107  i.p. 30 min prior to 3 Gy, Days 1-7.
Figure 3

A

![Graph showing CPM (counts per minute) for DMSO, 25 μM, and 50 μM treatments.](image)

B

![Images showing fluorescence under DMSO and YTR107 treatments.](image)

C

![Western blot analysis for Chk1 (Ser 317), Chk1 total, and GAPDH.](image)

D

![Western blot analysis for γ-H2AX (Ser-139) and GAPDH.](image)
The novel chemical entity YTR107 inhibits recruitment of nucleophosmin to the sites of DNA damage, suppressing repair of DNA double strand breaks, and enhancing radiosensitization.


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