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Replication Stress Induced by the Ribonucleotide Reductase Inhibitors Guanazole, Triapine, and Gemcitabine in Fission Yeast

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REPLICATION STRESS INDUCED BY THE RIBONUCLEOTIDE REDUCTASE INHIBITORS GUANAZOLE, TRIAPINE, AND GEMCITABINE IN FISSION YEAST

A thesis submitted in partial fulfillment of the
requirements for the degree of
Master of Science

By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Mashael Yahya A Alyahya ENTITLED Replication Stress Induced by the Ribonucleotide Inhibitors Guanazole, Triapine, and Gemcitabine in Fission Yeast BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Alyahya, Mashael Yahya A. M.S., Department of Pharmacology and Toxicology, Wright State University, 2022. Replication Stress Induced by the Ribonucleotide Inhibitors Guanazole, Triapine, and Gemcitabine in Fission Yeast.

Replication stress can be produced by various exogenous or endogenous factors that perturb the movement of replication forks. To overcome the stress, eukaryotic cells activate the DNA replication checkpoint to mobilize several pathways to protect the forks, maintain genome integrity, and promote cell survival. Defects in the replication checkpoint cause forks collapse, leading to chromosomal DNA damage or cell death. Although the replication checkpoint is crucial for genome integrity in all eukaryotes, the underlying mechanisms remains to be fully understood. To investigate the mechanisms of the replication checkpoint, hydroxyurea (HU), an established inhibitor of ribonucleotide reductase (RNR), has been widely used in laboratories as an inducer of the replication stress. It depletes dNTP pools, slows down the movement of DNA polymerase at the forks, and thus activates the replication checkpoint in yeasts and in mammalian cells. Unfortunately, HU also targets other cellular components, which may complicate the studies, leading to ambiguous description of the checkpoint mechanisms. The purpose of this study is to find an RNR inhibitor that produces the replication stress more specifically than HU in the fission yeast *Schizosaccharomyces pombe*. We examined three RNR inhibitors, namely guanazole, triapine, and gemcitabine under several experimental conditions that are commonly used in the laboratories for checkpoint

studies. We found that among the three drugs, guanazole and triapine produce the replication stress more specifically than HU under the chronic drug exposure conditions such as spot assay. Under acute drug treatment conditions, however, guanazole and triapine cause other cellular stresses more significantly than HU. Therefore, using guanazole or triapine in chronic drug exposure conditions and HU in acute treatment can produce replication stress specifically under various experimental conditions and thus benefits the checkpoint studies in *S. pombe* and possibly the research in other model systems.

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DRC	DNA replication checkpoint
DDC	DNA damage checkpoint
ssDNA	Single-stranded DNA
DSBs	Double strand breaks
ATR	Ataxia telangiectasia and Rad3 related
ATRIP	ATR interacting protein
Mrc1	Mediator of replication checkpoint 1
RNR	Ribonucleotide reductase
dNTPs	Deoxynucleotide triphosphates
HU	Hydroxyurea
GZ	Guanazole
3-AP	Triapine
GEM	Gemcitabine
S phase	DNA synthesis phase
G2/M	Growth phase 2/Mitosis
S. pombe	*Schizosaccharomyce pombe*
TCA	Trichloroacetic acid

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1. INTRODUCTION

1.1 Eukaryotic cell cycle and DNA Replication

A key characteristic for life is the ability of a cell to divide into two identical daughter cells through a programmed mechanism known as cell cycle (1). Although fission yeast and human cells are all eukaryotic, there are differences in their cell cycles. One complete cell cycle of fission yeast takes about 3 hours. The growth phase 2 (G2) is the longest phase where it consumes about 70% of the cell cycle time while growth phase 1 (G1), replication (S phase), and mitosis contribute to the remaining 30% in fission yeast (2). Also, one important feature of *Schizosaccharomyces pombe* (*S. pombe*) is the initiation of replication even before the cytokinesis finishes. Although DNA damage can occur in any stage of the cell cycle, the cells are susceptible to the threats particularly during DNA replication (3). During DNA replication, the double-stranded DNA helix unwinds, employing each parent strand as a template to synthesize the nascent strand by DNA polymerase (4). An accurate replication process can be interrupted by a variety of endogenous and exogenous factors, which generates replication stress (3).

1.2 DNA replication stress

Replication stress threatens the genome stability and cell viability. The stress is a state where the fork movement is slowed or stalled. It usually does not indicate the physical damages to the DNA structure such as double stranded breaks (DSBs) that resulted from collapsed forks; however, it may generate a physical structure known as extended stretches of single-stranded DNA (ssDNA) (5). The ssDNA usually arises because the helicase proceeds to unwind the parent strands even in the presence of slowed or halted DNA polymerase (6). In fact, the replication stress can be generated under various circumstances, for instance, depletion or imbalance of the dNTP pools, the precursors of DNA synthesis, lesions on the DNA templates, replication-transcription conflicts, and lacking vital replication factors such as histones (5). Under these conditions, the activated DNA replication checkpoint (DRC) deals with the stress by stimulating dNTP production, preventing forks from collapse, and promoting cell survival.

1.3 The DRC signaling pathway

The DRC is a cell cycle surveillance system evolved in eukaryotic cells to preserve genome stability by protecting the stressed forks and arresting the cell cycle to allow time for the DNA replication to be completed (7). Upon formation of ssDNA at the

slowed or stalled forks under stress, replication protein A (RPA) complex, composed of RPA70, RPA32, and RPA14, binds to ssDNA to prevent its degradation (7,8). The ssDNA-RPA complex works as a platform for recruiting Rad3 (ATR in mammals) and Rad26 (ATRIP in mammals). In addition to Rad3 and Rad26, ssDNA-RPA promotes the loading of a ring like sliding clamp called 9-1-1 complex composed of Rad9, Rad1, and Hus1 onto the forks by the loader Rad17 and the replication factor C (RFC2-5) complex (8).

Once the assembly of the checkpoint sensor proteins (Rad3, Rad26, Rad9, Rad1, and Hus1) is completed at the fork, Rad3 is activated to phosphorylate the neighboring Rad3 proteins in trans to intensify the signaling response, leading to full activation of the DRC (7). In addition to the DRC, Rad3 also launches a signaling cascade of the DNA damage checkpoint (DDC) pathway. When ssDNA generation occurs in S phase, the DRC pathway is activated by Rad3-dependent phosphorylation of the mediator of replication checkpoint Mrc1 and the effector kinase Cds1 (Claspin and Chk2 in mammals, respectively) (9). When DNA damage occurs outside the S phase or in the case of collapsed forks in DRC mutants, Rad3 phosphorylates Crb2 (53BP1 in mammals) and Chk1 at the damage sites to initiate the DDC. The activated DRC suppresses the firing of late replication origins, increases the production of dNTPs by upregulating the R2 subunit of ribonucleotide reductase (RNR), safeguards and restarts replication forks, prevents fork collapse and thus, prevents replication catastrophe and cell death (7,9,10). Defects in

DRC are linked with mutagenesis and genetic diseases (4,11). Since the checkpoint proteins are highly conserved from yeasts to humans throughout evolution (6), studying the checkpoint using fission yeast as a model will provide further insights into the checkpoint mechanisms.

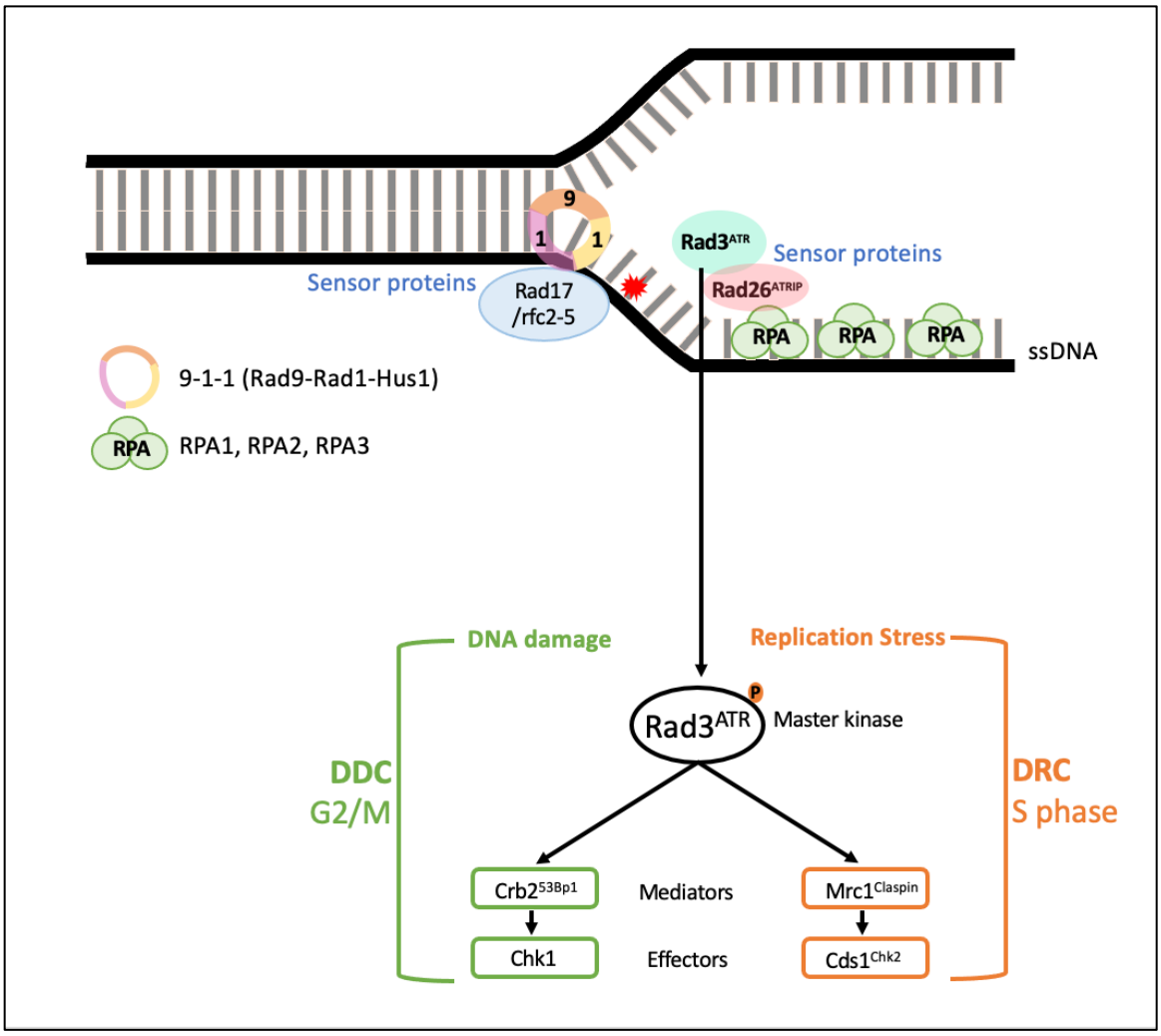

Figure 1: The current model of the DRC and DDC signaling pathways

When a replisome encounters a DNA lesion on the template, it leads to the slowing of DNA polymerase movement, but not the unwinding helicase, generating ssDNA. The resulting ssDNA binds RPA to form a complex which work as a platform to recruit the sensors kinases Rad3 and Rad26 (ATR and ATRIP in human cells) (7). Another sensor proteins complex termed as 9-1-1 (Rad9, Rad1, and Hus1) is assembled at the stalled replication fork by the loader Rad17 and Rfc2-5 (8). When the checkpoint sensors are assembled at the fork, Rad3 undergoes autophosphorylation and transmits the signal to the mediator kinase Mrc1(Claspin in mammalian cells) (3). Then, Mrc1 mediates the downstream signal transduction to the effector kinase Cds1 (Chk2 in humans) of the DRC (9). If DNA damage occurs outside the S-phase of the cell cycle or in the presence of collapsed forks which generate double strand breaks, Rad3 phosphorylates and activates Crb2 (BRCA1 in mammals) and Chk1 of the DDC pathway (3).

1.4 Activation of the DRC

Upon activation of the DRC, several mechanisms take place to overcome the replication stress. These include increasing dNTPs levels, slowing the replication progression, inhibiting the late firing origins, stabilizing the perturbed forks, and preventing entry into mitosis. All these factors work in concert to maintain genome stability and cellular proliferation. (12–15).

Although it is important for genome integrity, the DRC mechanism remains incompletely understood. As illustrated earlier, DNA replication process could be hindered by a variety of factors that generate replication stress. Some of the factors include reactive oxygen species (ROS), ultraviolet light (UV), and methyl methanesulfonate (MMS) that generate DNA damage (3,5). Although these DNA damaging agents can activate the DRC, it also activates the DDC if the DNA damaging effect occurs in G2 phase of the cell cycle, leading to a vague explanation of the DRC mechanisms (16). Therefore, an agent that specifically produces replication stress is critically important for the studies of the DRC.

1.5 Hydroxyurea

Hydroxyurea (HU) is a simple compound discovered in 1869 by Dresler and Stein that was reported to have a potential antineoplastic activity in 1960's (17). Since then, it has been used for a wide range of conditions including sickle cell anemia, viral infections, psoriasis, and several types of cancer (18,19). Taking advantages of its

cytotoxic effect, nowadays, it is used to treat skin cancer, chronic myeloproliferative disorders, and many solid tumor types (18,20,21). The profound effect of HU was noted in 1964 when it was found to inhibit RNR by quenching the tyrosine free radical, which is required for the catalysis (22). It is the most widely used agent in laboratories to induce the reversible replication stress and S phase cell synchronization. (22,23). Although it is used to induce replication stress, HU may provoke DDC under different circumstances. For example, if a DNA damage such as broken forks occur, when HU is used in high doses or for long term exposure.

1.6 RNR, the primary target of HU.

The activated DRC inhibits cell division to allow time for DNA replication and repair. Furthermore, it upregulates RNR to enhance dNTPs production. RNR is an enzyme responsible for generating the four deoxynucleotide triphosphates (dNTPs), the building blocks of DNA, by substituting the 2'-hydroxyl group of dNDPs with a hydrogen atom (24,25). By regulating production of dNTPs, RNR tightly controls mutational rate, therefore, enhances the DNA replication and repair (26). There are three classes of RNR, I, II, and III. Among these, class I RNR is found in mammals, eukaryotes and prokaryotes. RNR consists of two subunits in a dimeric form, the larger R1 (α) subunit and the smaller R2 (β) subunit (Fig.2). Nucleotides are catalyzed in R1 subunit where it contains the active reduction site. R1 also involves two allosteric sites, the first

site regulates the substrate specificity while the second site binds to ATP for enzymatic activation or to dATP for inhibition. On the other hand, R2 which is highly expressed during S-phase, harbors the diferric-oxygen center that yields the stable tyrosyl free radical which travels to R1 via a long chain radical transfer pathway. Then it reduces cysteine residues to produce thiyl radical which is responsible for RNR activity. HU deactivates the tyrosyl radicals and thus inhibits RNR. This inhibition generates replication stress and activates the DRC in yeasts and mammalian cells. (21,24,26–28).

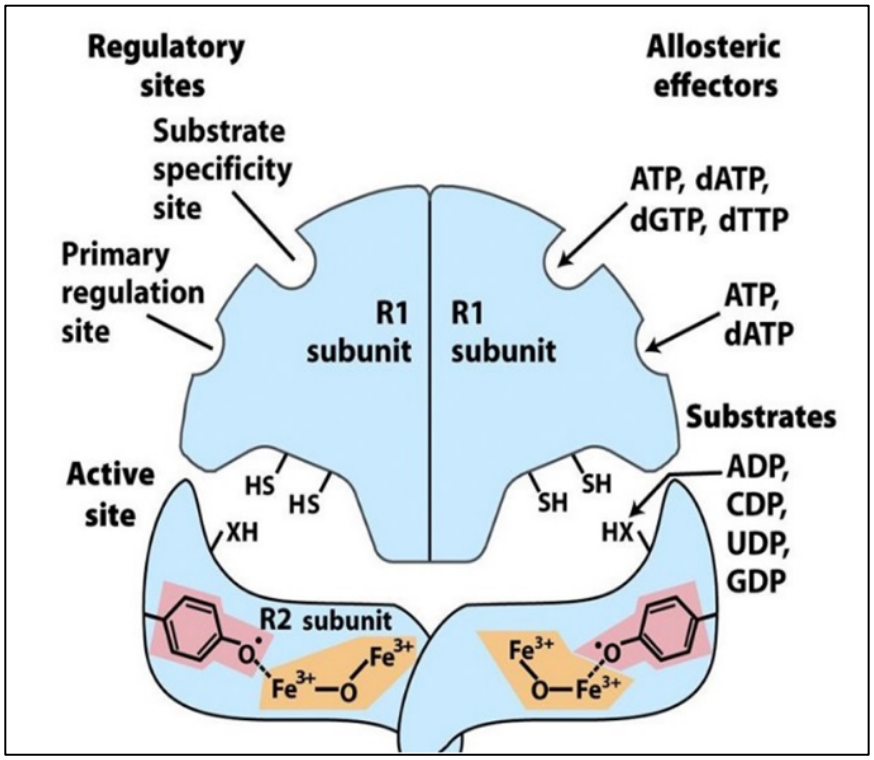

Figure 2: The Structure of Ribonucleotide Reductase

The enzyme consists of two subunits, R1 and R2. R1 regulates the dNTPs production and R2 is essential for enzymatic activity through the presence of the tyrosyl free radicals (29).

1.7 The secondary targets of HU

Extensive studies have been conducted to better understand the cell-killing mechanisms of HU. As mentioned earlier, HU inhibits RNR, and thus, activates the DRC (10,30). Mutants with a defective DRC are much more sensitive to HU than wild type cells (10,14,31). However, previous studies revealed that some mutants that are sensitive to HU possess an intact DRC (14,31). Since the DRC remains functional, the cytotoxic effect of HU is unrelated to RNR inhibition. These mutants, namely *hem13-1* and *erg11-1*, have defects in heme and ergosterol pathways, respectively. They manifest the cell killing effect of HU by oxidative stress or cytokinesis arrest but not RNR inhibition (14,31). These findings offer a deeper understanding of the cell killing effect of HU (32).

To see whether HU has other potential cellular target(s), further characterizations of these mutants have taken place. *Erg11* is a gene encoding sterol-14α-demethylase essential for ergosterol biosynthesis and one study found that a single missense mutation causing the G189D amino acid substitution in *erg11* in *S. pombe* which sensitizes the cell to HU (31). This mutation contributes to the lower ergosterol levels, and the cytokinesis arrest induced by HU (31). It has also been reported that a T263I mutation in *hem13*, a gene encoding coproporphyrinogen III oxidase required for heme biosynthesis, is attributed to the HU teratogenic effect. The generation of ROS in the form of hydroxyl radical is believed to be the underlying killing mechanism of HU in the *hem13* mutant. (14). However, both metabolic mutants have shown only a chronic (~3 days) but not an

acute sensitivity (~3 hours) to HU, which supports the notion that the cytotoxic effect of HU is independent of RNR inhibition in these mutants.

This newly uncovered cell killing mechanisms of HU, although important for HU-based chemotherapies, may compromise the checkpoint studies. In this study, we tested three RNR inhibitors namely guanazole, triapine, and gemcitabine, aiming to find an agent that can produce replication stress more specifically than HU. Such an agent, once identified, will be very helpful to the checkpoint studies, and that can lead to the development of new chemotherapeutic regimens.

1.8 RNR Inhibitors examined in this study

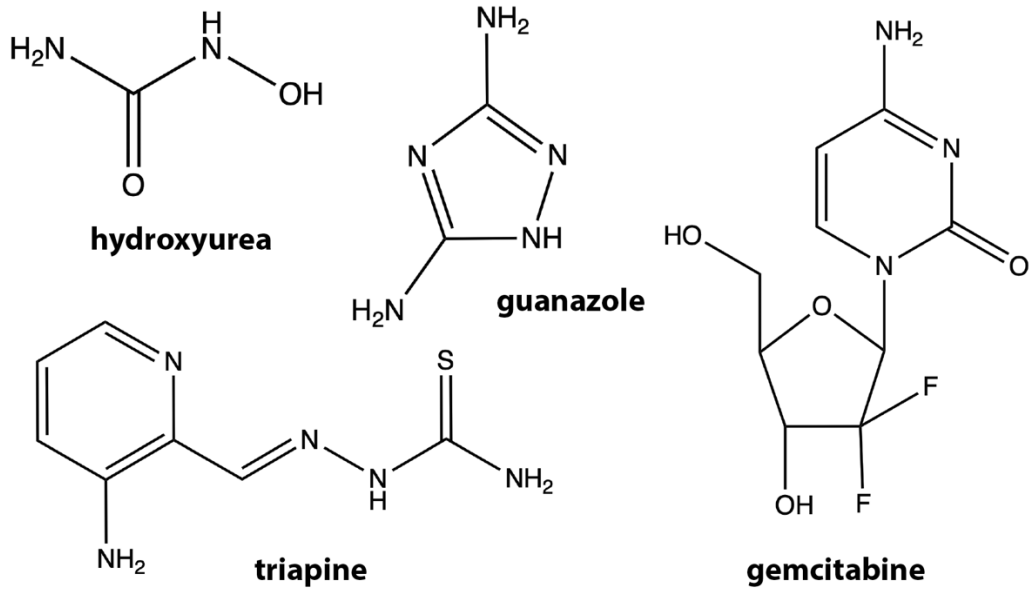

Figure 3: Chemical Structures of hydroxyurea, guanazole, triapine, and gemcitabine

1.8.1 Guanazole

Although guanazole (GZ) (Fig.3) was synthesized in 1893, its biological action wasn't reported until 1970 as antineoplastic for leukemia in mice (33). A phase I clinical study on 27 patients with acute leukemia revealed a complete remission of two patients and reported that myelosuppression is the main side effect (34). Thereafter, the interest in GZ as a clinical agent for cancer treatment has been reduced since phase II trials were never initiated. Another study reported that GZ and HU have the same DNA synthesis and ribonucleotide effects in leukemia L1210 cells (33). Like HU, GZ inhibits the DNA

synthesis by suppressing RNR activity. However, it is less genotoxic and cost-effective, which makes it superior to HU (33,35).

1.8.2 Triapine

Triapine (3-AP) belongs to the α-N-heterocyclic thiosemicarbazones class, and it functions as a potent RNR inhibitor. It is 1000-fold more potent than HU in terms of RNR inhibition. Currently, it is being tested in phase II clinical trials as a promising antitumor compound (28,36). Shao J's group suggested that 3-AP doesn't have a direct effect on the tyrosyl radical. Instead, it forms an active redox complex with iron to generate ROS which destroy the tyrosyl radical (37). 3-AP has higher affinity to RNR than HU, however, the action of both drugs is limited to the S phase of the cell cycle (38).

1.8.3 Gemcitabine

Gemcitabine (GEM) is a cytidine analogue. It was discovered as an antiviral, and later as a cancer chemotherapeutic agent for the treatment of a wide range of tumors. Currently, GEM is indicated as a first line treatment for pancreatic cancer, and in combination with other anticancer drugs, it is also used in treating breast and non-small cell lung cancer (39). GEM manifests a cell-cycle specificity in cells experiencing DNA synthesis (40). It is transported into cells by a transporting system consisting of five nucleoside transporters (41). It is a prodrug that it is converted intrinsically into the active di- and triphosphate nucleosides by deoxycytidine kinases (40). The two metabolites inhibit the synthesis of DNA by dual cytotoxic mechanisms. The diphosphate derivative

inhibits RNR, thus, decrease the dNTP levels including dCTP. While the triphosphate nucleoside competes with dCTP for DNA incorporation (40). As a result, the low level of the intracellular dCTP promotes integration of gemcitabine diphosphate into DNA and thus, further inhibiting DNA synthesis (42,43). Earlier studies suggest that GEM causes a complete S phase arrest, which leads to a maximum checkpoint activation (44).

1.9 *S. pombe*, the model organism

The fission yeast *S. pombe* is used as the model system in this study. It is a unicellular, rod-shaped eukaryotic cell which was first isolated from millet beer in east Africa (45). The pure culture of *S. pombe* was made by Lindner and colleagues in the 1890s (45). Lindner called it *Schizosaccharomyce* because it replicates by fission, and *pombe* is the Swahili word for beer. It consists of three fully sequenced chromosomes with a majority of the genes being conserved in higher eukaryotes (45,46). *S. pombe* grows easily and exponentially in every three hours. So far, all checkpoint proteins have known homologues in *S. pombe,* which makes it an ideal model to study the checkpoint mechanism. We hypothesized that some of the tested RNR inhibitors would cause replication stress and activate the DRC more specifically than HU in *S. pombe*.

2. SPECIFIC AIMS

1. To examine the chronic and acute cell-killing effects of GZ, 3-AP, and GEM.

2. To assess the replication checkpoint activation by detecting the phosphorylation of Mrc1.

3. To analyze the cell cycle progression.

4. To study cellular morphology via microscopic analysis.

3. MATERIALS AND METHODS

3.1 Yeast Strains and Chemicals

Standard methods and genetic techniques were used for the yeast cell culture (47). Yeast strains used in this study are listed in Table. 1. The media used for cell culture was YE6S (0.5% yeast extract, 3% glucose, and the six essential supplements, adenine, uracil, histidine, lysine, leucine and arginine). Stock solution of 1.0 M GZ (AlfaAesar, Ward Hill, MI or Sigma-Aldrich, St.Louis, MO) was prepared by dissolving 9.91g in 100 mL of YE6S medium. GEM (AmBeed, Arligton Hts, IL) was prepared by dissolving in YE6S medium. 3-AP (ApexBio Tech LLC, Boston, MA) was dissolved in DMSO. HU (Sigma-Aldrich, St.Louis, MO) was dissolved in sterile deionized water.

3.2 Drug sensitivity

The sensitivities were tested by spot assay, acute spot assay, and colony recovery assay. For the spot assay or chronic drug exposure, the logarithmically growing *S. pombe* culture was collected in 1 OD/ml (or $2x10^7$ cells/ml), diluted in fivefold steps, and spotted on YE6S plates containing HU, GZ, GEM or 3-AP at the indicated concentration. The YE6S plates without the drugs were used as control. As the control plates for 3-AP, equal amount of DMSO was added. The plates were incubated at 30°C for 3 days and then scanned.

For examining the acute drug sensitivity, spot assay and the colony recovery assay were performed. For the acute spot assay, HU, GZ, or 3-AP were added to logarithmically

growing cultures. 0.5 OD of cells was removed every two hours, washed once with deionized water, diluted ten times in water, and spotted on YE6S plates. The plates were incubated at 30°C for 3 days and photographed. In the colony recovery assay (48), a sample of the culture was collected every hour during the drug treatment, diluted 1000-fold in deionized water, spread onto YE6S plates, and incubated at 30°C for the cells to recover for 3 days. The colonies were counted and plotted as percentages of the survival rate. Each data point represents the log of the average cell survival percent calculated from three separate plates. Error bars represent standard deviations of triplicates.

3.3 Western Blotting

Phospho-specific antibody against phosphorylated Mrc1-Thr^{645} was generated using the chemically synthesized phosphopeptides described in our previous studies (9,49). The fission yeast cells were fixed in 15% trichloroacetic acid (TCA) on ice overnight and lysed by a mini-bead beater. The lysate was separated on an 8% gel by SDS-PAGE and transferred to nitrocellulose membrane for western blot examination. The membrane was stained with Ponceau S and was used as a loading control. After extensive washing with distilled water, the blot was blocked with 5% milk and probed with the phospho-specific antibody in 5% milk solution for 3 hours to detect the phosphorylated Mrc1. The blot was then treated with secondary antibody. The same blot was stripped by incubating it at 70°C in a buffer composed of 50mM Tris:HCl (pH 7.5), 50mM DTT, and 2% SDS, washed extensively with distilled water, and reprobed with polyclonal antibody against

Mrc1 to show the protein levels. Using ChemiDoc XRS imaging system (Bio-Rad), the blotting signals were detected by electrochemiluminescence. The band intensities were quantified using ImageLab (Bio-Rad).

3.4 Flow cytometry

0.5 OD cells were collected by centrifugation and fixed overnight in 70% ice-cold ethanol at 4°C. The fixed cells were treated with 50 mM sodium citrate containing 0.1 mg/ml RNase A at 37°C for ≥ 3hr. The cells were stained with 4 μg/ml propidium iodide (PI), extensively vortexed and analyzed by Accuri C6 flow cytometer using FL2-A channel. FCS Express 4Flow software was used to analyze the data.

3.5 Microscopy

S. pombe cells were incubated with either 15 mM HU or 200 mM GZ for 3 hours at 30°C. 0.5 OD cells were centrifuged, washed once with PBS, and centrifuged again. About 2 μl of cells was removed directly from the pellet, placed onto a glass slide, and heat-fixed briefly at 70°C. The fixed cells were stained with Blankophor (1:50 dilution in PBS) and Hoechst (1:50 dilution in PBS). The cells were visually examined using an Olympus EX41 fluorescent microscope. Images were captured with an IQCAM camera (Fast1394) using Qcapture Pro 6.0 software and exported to Adobe Photoshop to create the figure.

Table 1: List of S. pombe strains used in the study

Strain	Genotype	Source
TK48	*h⁻ leu1-32 ade6-M216*	Kelly lab
NR1826	*h⁻ Δrad3::ura4 leu1-32 ura4-D18 ade6*	Russell lab
TK197	*h⁺ Δchk1:ura4 leu1-32 ura4-D18 ade6-M210*	Kelly lab
YJ15	*h+ mrc1:ura4 leu1-32 ura4-D18 ade6-M210*	Lab stock
GBY191	*h⁺ Δcds1:ura4 leu1-32 ura4-D18 ade6*	Lab stock
Erg11-1	*h⁺erg11(G189D): ura4+ leu1-32 ura4-D18 ade6-M210*	Lab stock
APS19	*h⁺ hem13(T263I)-1 cds1-6his2HA(Int) leu1-32 ura4-D18 ade6-M210*	Lab stock

4. RESULTS

4.1 Sensitivity of *S. pombe* to GZ, GEM, and 3-AP determined by spot assay.

The cytotoxic effect of GZ, GEM, and 3-AP was determined by the spot assay. Spot assay is simple, fast and has been widely used in genetic screening. In this experiment, HU was included for comparison. To assess the replication stress induced by the RNR inhibitors, the following *S. pombe* strains were used: wild type (TK48), the checkpoint mutant cells *rad3*Δ (NR1826), *mrc1*Δ (YJ15), *chk1*Δ (TK197), and *cds1*Δ (GBY191), and two metabolic mutants *erg11-1* (YJ1296) and *hem13-1* (APS19) (14,31) (Table1). The wild type *S. pombe* cells were used as a control, whereas *rad3*Δ*, mrc1*Δ, *chk1*Δ*,* and *cds1*Δ mutants were used to examine the checkpoint response to the induced replication stress. Because *erg11-1 and hem13-1* are sensitive to HU due to other stresses such as cytokinesis arrest and oxidative stress (14,31), they are used as indicators for other potential stresses. As mentioned previously, $Rad3^{ATR}$ is the sensor kinase for both the DRC and the DDC. *S. pombe* lacking Rad3 is highly sensitive to the replication stress and the DNA damage due to a lack of the DRC and the DDC. However, mutants of the Rad3-Mrc1-Cds1 (DRC pathway)*,* but not Rad3-Crb2-Chk1 (DDC pathway), are highly sensitive to the replication stress.

As shown in Fig. 4, top panel, *rad3*Δ*, mrc1*Δ*,* and *cds1*Δ cells showed a sensitivity to HU, whereas *chk1*Δ mutant is less sensitive, indicating that the replication

stress induced by HU is mainly dealt with by the DRC, and not the DDC. However, *chk1*Δ cells showed sensitivity when HU was increased to 5 mM, suggesting that even in the presence of functional DRC, the DDC was also activated likely by the DNA damage resulting from the collapsed forks. Consequently, *mrc1*Δ and *cds1*Δ showed a sensitivity to HU while *rad3*Δ cells were more sensitive than *mrc1*Δ and *cds1*Δ which is consistent with the function of Rad3 in both DRC and DDC. Although *erg11-1* and *hem13-1* show a similar or even higher sensitivity than *rad3*Δ mutant in the spot assay (Fig. 4 lower section of the top panel), they are not sensitive to HU under the acute treatment in liquid culture for 8 hours, suggesting that in addition to the replication stress, HU causes other stresses leading to cell death of the metabolic mutants in the chronic spot assay (14,31). To find an agent that activates DRC more specifically, three RNR inhibitors GZ, GEM, and 3-AP were tested under similar conditions. As shown in Fig. 4 lower right panel, GEM doesn't have any growth inhibitory effect to all tested strains when up to 77.5 mM was used (results with lower concentrations are not shown). GZ and 3-AP caused a higher cytotoxic effect on *rad3*Δ and *cds1*Δ than *chk1*Δ and wild type *S. pombe*, suggesting that they produce replication stress. Furthermore, the *chk1*Δ mutant treated with 60 mM GZ is less sensitive than 5 mM HU, which suggests that the replication stress induced by GZ is mainly dealt with by the DRC. Notably, the two metabolic mutant strains were less sensitive to 15 mM GZ and 0.25 mM 3-AP than 1.25 mM HU under similar conditions. However, when the concentrations of GZ and 3-AP were increased, the metabolic mutants were also sensitive, which suggests the presence of

other cellular stress in addition to replication stress when higher doses were used (see below). Therefore, GZ and 3-AP can more specifically produce replication stress than HU in the spot assay. Between the two drugs, GZ is more cost-effective (about 30% less than HU), we recommend using GZ to generate the replication stress under chronic drug exposure conditions such as the spot assay. To exclude the possibility that the cell killing effect of GZ is due to the presence of impurities, GZ samples from two different manufacturers were compared side by side. Yet, the results were very similar, if not identical, suggesting that the observed cytotoxic effect is caused by GZ, and not the impurities associated with the samples.

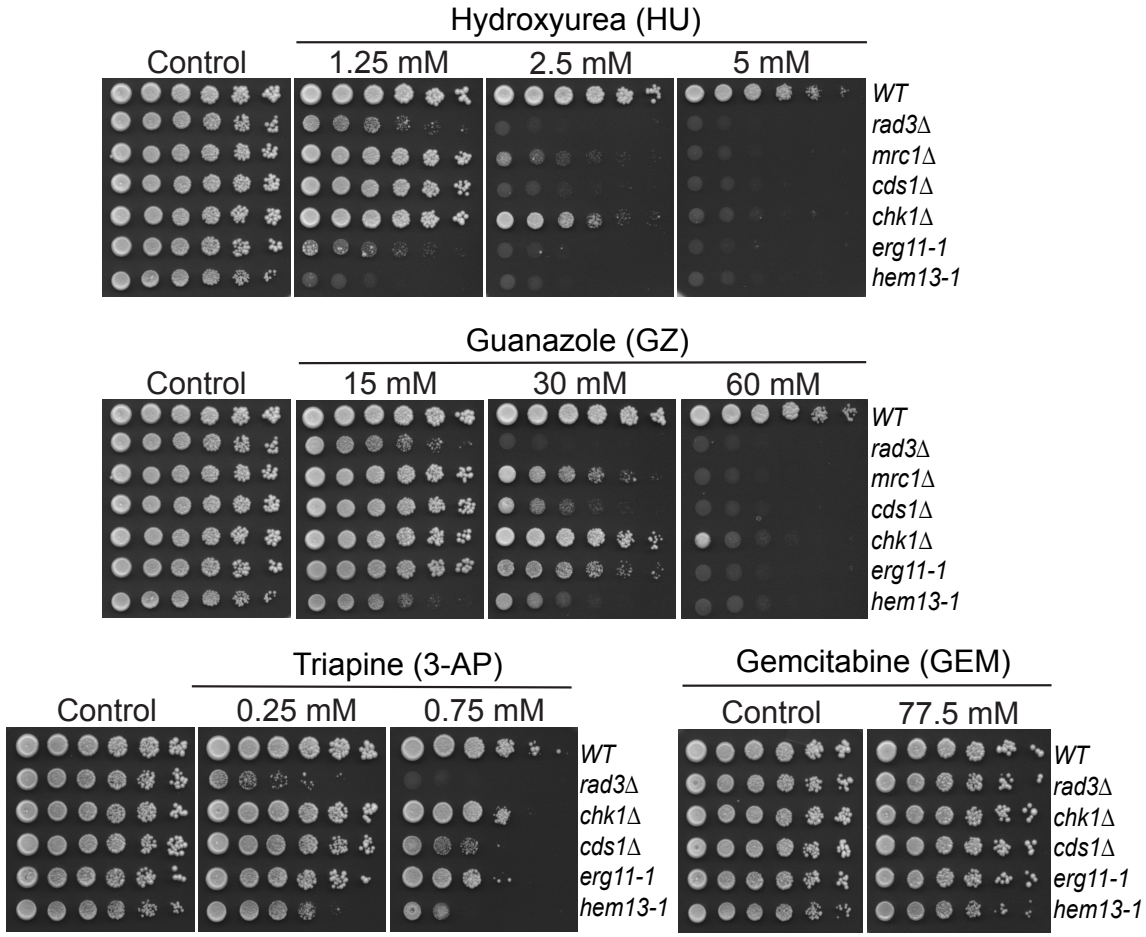

Figure 4: Chronic drug sensitivity of wild-type (WT), rad3Δ, chk1Δ, mrc1Δ, cds1Δ, erg11-1, and hem13-1 analyzed by spot assay

Fivefold dilution series of the logarithmically growing *S. pombe* were prepared and spotted on YE6S plates containing HU, GZ, GEM or 3-AP at the indicated concentrations. While the control plates contained only YE6S medium, the drug plates contained HU, GZ, and GEM. 0.5% of DMSO vehicle control was included in the non-

drug plates for 3-AP. The plates were then incubated in 30°C for 3 days and then photographed.

4.2 Acute cell-killing effect of GZ and 3-AP.

Based on the results by spot assay, we selected GZ and 3-AP to further investigate their inhibitory cell growth effects in liquid cultures. GEM was excluded from subsequent analysis because it does not show any growth inhibitory effect in *S. pombe*. To study the acute drug sensitivity in liquid medium, two methods were used: acute spot assay and colony recovery assay.

In the acute spot assay, GZ and 3-AP were added to liquid YE6S cultures of wild type and *rad3*Δ cells at the indicated concentrations. For comparion, 15 mM HU was included in this experiment. While the cultures were incubated at 30 °C in the presence of the drugs, a small number of cells was removed every two hours during the drug treatment, washed once with deionized water, diluted ten times, and spotted on YE6S plates. To allow cell recovery, the plates were incubated at 30 °C for 3 days. As shown in Fig. 5A, HU showed the cytotoxic effect to *rad3*Δ but not the wild type cells after 2 hours of incubation. Under similar conditions, 3-AP did not show any cell-killing effect during the 8 hours of treatment in the presence of 5 mM or 7 mM concentration. This may be because 3-AP is transported into the cells very slowly, which prevents its cytotoxic effect during the acute treatment. However, GZ showed a toxicity to *rad3*Δ cells at

concentrations ranging from 50 mM to 250 mM (Fig. 5B). Based on these results, we conclude that unlike 3-AP, GZ is capable of inducing replication stress under the acute treatment conditions. Surprisingly, despite the concentrations of GZ were increased, the growth inhibition of *rad3*Δ did not increase accordingly and never reached the level of 15 mM HU. This result is consistent with the data in Fig. 4, suggesting that GZ may also generate another type of stress causing G2/M delay, which indirectly suppresses to some extent the cytotoxic effect caused by the replication stress in liquid cultures.

To further analyze the replication stress induced by GZ, we performed the colony recovery assay. In this assay, a 15 mM HU or 200 mM GZ was added to the liquid culture containing wild type, *rad3*Δ*, cds1*Δ*, and erg11-1* cells and incubated at 30 °C. Every hour during the treatment, a small amount of the culture was removed, diluted 1000 times, and spread onto three YE6S plates to recover. The plates were incubated at 30 °C for 3 days. The recovered colonies were counted and shown in percentages of the cell survival rate. As shown in Fig. 5C, both wild type and *erg11-1* cells exhibited a similar resistance to HU and GZ. Although *erg11-1* was sensitive to HU under chronic conditions (Fig. 4), it is resistant to the acute treatment, which confirms our previously published results (14,31). The *cds1*Δ mutated cells were less sensitive than *rad3*Δ in HU, which is consistent with the results shown in Fig. 4 and with the roles of Cds1 and Rad3 in the checkpoint pathways. As shown in Fig. 5C right panel, *cds1*Δ and *rad3*Δ were more sensitive than *erg11-1,* which indicates the presence of replication stress. In contrast

to HU, GZ sensitizes *rad3*Δ and *cds1*Δ cells in a similar pattern. Together, in addition to Fig. 4, these results show that on top of the replication stress, GZ may cause another stress during the acute treatment that suppress the cytotoxic effect in the *rad3*Δ cells.

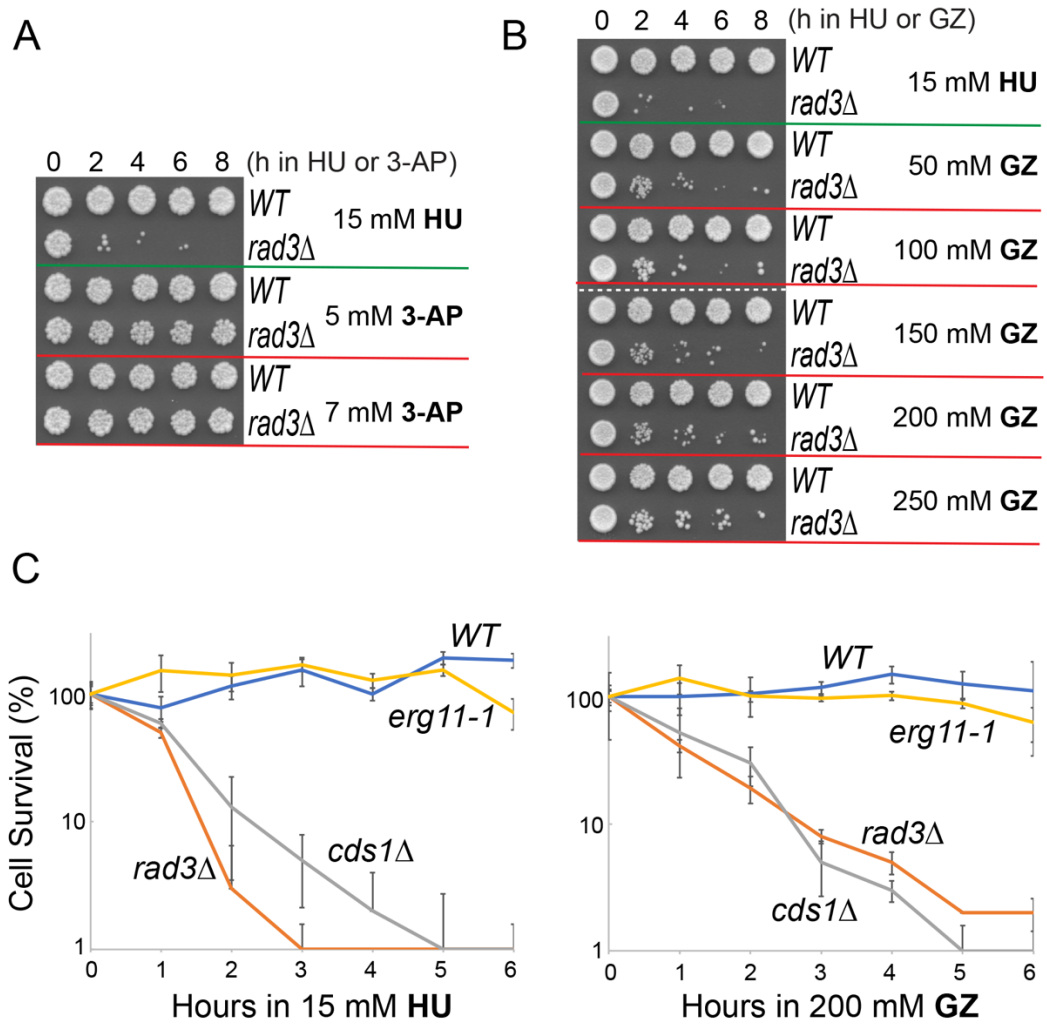

Figure 5: Acute drug sensitivity of wild type and rad3Δ cells analyzed by acute spot and colony recovery assays

(A) WT and *rad3*Δ cells were treated with 15 mM HU (green line), or 5 mM and 7 mM 3-AP (red lines). A small amount of the culture was removed every two hours during the treatment, washed once and spotted on YE6S plates. The plates were incubated at 30℃ and then photographed. **(B)** WT and *rad3*Δ mutant cells were treated with 15 mM HU (green line) or increasing concentrations of GZ (red lines). Dash line indicates discontinuity. (**C)** The sensitivity of WT, *erg11-1*, *rad3*Δ, and *cds1*Δ cells to HU and GZ was determined by cell recovery assay. The cells were treated with 15 mM HU (left panel) and 200 mM GZ (right panel) in YE6S liquid medium. The cells were spread onto YE6S plates every hour during the drug treatment and incubated at 30°C for three days to recover. The colonies were counted and plotted as percentage against time. Error bars represent standard deviations of triplicates.

4.3 Effect of GZ on Rad3 kinase signaling in the DRC.

Next, we examined the phospho-signaling of DRC to confirm the presence of replication stress induced by GZ. Considering that 3-AP did not show any acute cytotoxic effect, it was not further studied. In the presence of replication stress, Rad3 phosphorylates two Mrc1 residues, Thr^{645} and Thr^{653} that can be monitored by western blotting using the phospho-specific antibody as explained in our previous studies (9,10,49). The phosphorylated Mrc1 facilitates signals transmission from Rad3 to Thr^{11} residue in Cds1, which encourages the autophosphorylation of Thr^{328} and the

autoactivation of Cds1, the effector kinase of the DRC (9). Hence, Mrc1 is a molecular indicator of the activated DRC and of the presence of replication stress.

In Fig. 6A, Mrc1-Thr^{645} phosphorylation was examined in wild type cells treated with increasing concentrations of GZ. When the concentration of GZ increased, the levels of Mrc1 phosphorylation were also increased accordingly until it reaches a plateau (Fig. 6B, blue line). Consistent with the results shown in Fig 4 and 5, 200-300 mM of GZ stimulates Mrc1 phosphorylation at levels comparable to that in cells treated with 15 mM HU. Mrc1 is expressed specifically in S-phase (9) and the activated DRC promotes the expression of Mrc1 (50). Therefore, the Mrc1 protein level is much higher in GZ-treated cells than in untreated cells (Fig. 6B, orange line). Interestingly, Mrc1 phosphorylation decreased when the concentrations of GZ were higher than 150 mM. These data together with that shown in Fig 4 and 5 suggest the presence of another type of stress apart from the replication stress, causing G2/M arrest (see below). The G2/M arrest has been shown in *erg11-1* and *hem13-1* cells when a higher dose of HU was used, which indirectly affects the levels of Mrc1 protein and its phosphorylation (21,31).

To investigate if the Mrc1 phosphorylation is dependent on Rad3 in GZ-treated cells and if the phosphorylation occurs in *erg11-1* cells, western blot analysis was performed in cells treated with 15 mM HU or 200 mM GZ. As shown in Fig. 6C, Mrc1 phosphorylation levels in GZ-treated cells were higher than the control but lower than HU-treated cells. Notably, in *rad3*Δ and *mrc1*Δ cells treated with either HU or GZ, the

Mrc1 phosphorylation was eliminated. This is consistent with the signaling cascade of the DRC pathway which is controlled by the master checkpoint kinase Rad3. In other words, the phosphorylation of Mrc1 in the presence of GZ is also dependent on Rad3, which supports the acute induction of replication stress by GZ *in vivo*. As explained earlier, *erg11-1* mutant showed a G2/M cell cycle arrest in HU. Thus, upon the treatment of *erg11-1* with HU, Mrc1 phosphorylation levels was lower than in wild type cells. Likewise, Mrc1 phosphorylation levels in GZ-treated *erg11-1* cells were also lower than in wild type cells. Overall, these results strongly imply that higher doses of GZ induce another stress, causing a G2/M cell cycle arrest (see below).

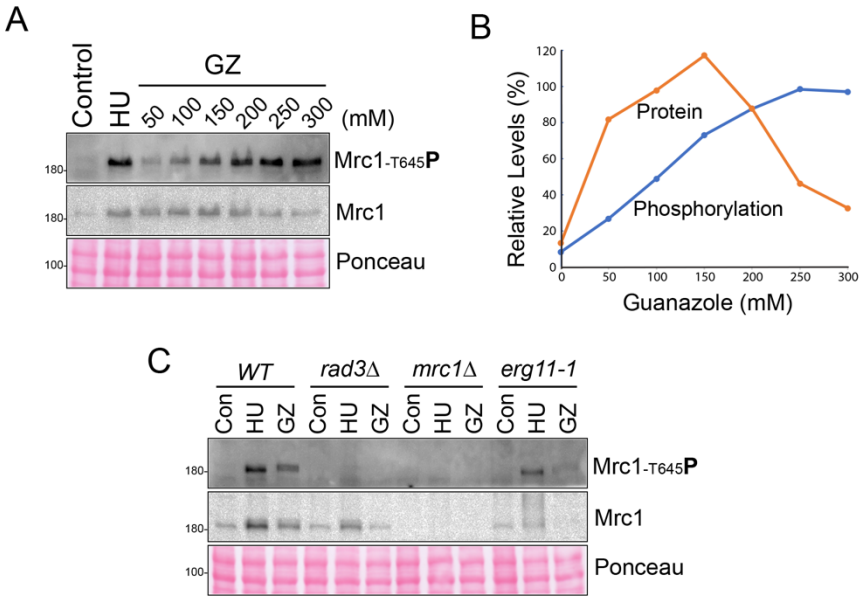

Figure 6: HU or GZ-induced Rad3-dependent Mrc1 phosphorylation

(A) Mrc1 phosphorylation in the presence of GZ or HU. Wild-type cells were treated with 15 mM HU or increasing concentrations of GZ for 3 hours. An equal number of cells were fixed in 15% TCA and lysed by mini-bead beater method. The whole cell lysate was separated on 8% SDS PAGE for Western blot analysis using the phospho-specific antibody (top panel). In the middle panel, the same blot was stripped and reprobed with anti-Mrc1 antibodies for Mrc1 protein level detection. A section of Ponceau S stained membrane was shown as the loading control (bottom panel). **(B)** The bands from the blot shown in A were quantified and represented as percentages of relative levels of protein (orange line) and phosphorylation (blue line) of Mrc1. **(C)** The WT and the indicated *S. pombe* mutant cells were treated with 15 mM of HU or 200 mM of GZ for 3 hours and analyzed as in (A).

4.4 Effect of GZ on cell cycle progression.

The results described above suggest that in addition to replication stress, GZ may also induce another stress, which perturbs the cell-cycle progression. To investigate this possibility, cell-cycle analysis was carried out in the presence of 200 mM GZ. Two sets of controls, wild type and *rad3*Δ treated with 15 mM HU were included in this experiment. At every hour time point of the drug treatment, 0.5 OD cells were removed and fixed for flow cytometry analysis. The results showed that the majority of the cells show a 2C content at 0 hour (Fig. 7, red line) because the S phase is very brief, and the

DNA replication starts before the completion of mitosis. It is also shown that both the wild type and *rad3*Δ cells were arrested by HU at the S-phase during the first 3 hours of incubation (Fig. 7, green line). However, when treated with GZ, the cells were arrested in G2/M phase during the 6 hours of treatment (Fig. 7 red line). This G2/M arrest aligns with the molecular evidence shown in Fig. 6B that while GZ causes replication stress, the majority of *S. pombe* cells were arrested at the G2/M phase, not at S-phase, suggesting the presence of another type of stress that arrests the cells at G2/M particularly when higher concentrations of GZ were used. This side effect of GZ, which may function similarly as the DNA damage checkpoint, explains the reduced sensitivity of *rad3*Δ mutant to GZ in Fig. 5 as well as the reduced expression in the protein and phosphorylation levels of Mrc1 in wild type cells shown in Fig. 6.

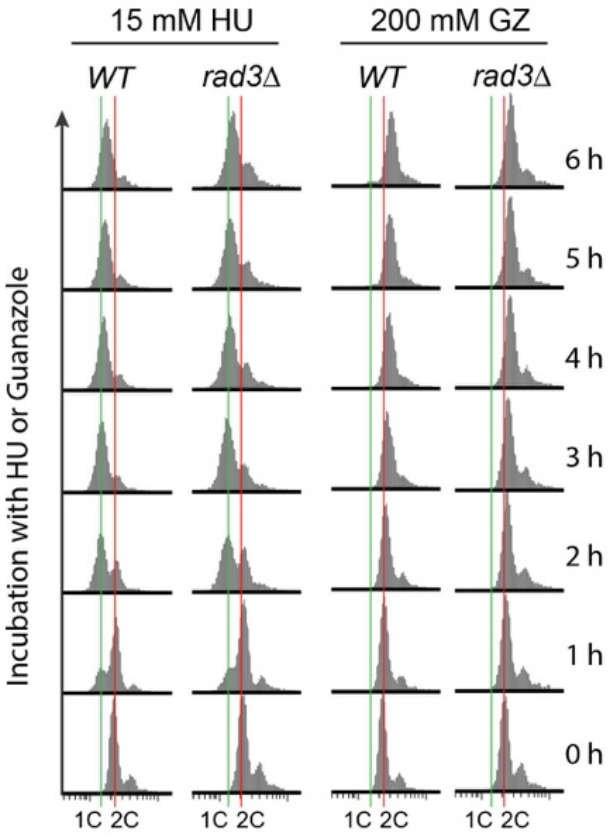

Figure 7: Cell cycle analysis of wild type and rad3Δ cells treated with HU or GZ examined by flow cytometry

HU or GZ was added to wild type or *rad3*Δ cultures at the indicated concentrations. Every hour during the incubation, an equal number of cells was collected and fixed with 75% ethanol. The fixed cells were then treated with RNase and propidium iodide for flow cytometry analysis. The green and red lines represent 1C and 2C DNA content, respectively.

4.5 Microscopic examination of cells treated with GZ

Replication stress is dealt with by the DRC. As mentioned above, the activated DRC mobilizes several cellular functions to ensure genome integrity and the viability of the cells. One of the cellar functions involves suppressing mitosis so that the cells have enough time to complete the DNA replication before cell division. However, *S. pombe* cells with a defective DRC such as *rad3*Δ mutant proceed into premature or aberrant mitosis in the presence of replication stress. This phenotype is called "cell untimely torn" or *cut* (51) and it is likely the direct cause for cell death in HU. Therefore, the *cut* phenotype is a strong indicator of the DRC defect in the presence of HU-induced replication stress. However, acute HU treatment causes cytokinesis arrest in *erg11-1* cells, not the *cut* cells, suggesting the side effect of HU in this mutant (31). To further investigate the replication stress and the side effect of GZ, we examined the *cut* phenotype and cell morphology under microscope in GZ treated *S. pombe*. For this purpose, we treated wild type, *rad3*Δ, *mrc1*Δ, and *erg11-1* cells with 200 mM GZ or 15 mM HU for 3 hours. The cells were fixed by brief heating, stained with Hoechst for genomic DNA and Blankophor for septum, and examined under microscope.

As shown in Fig. 8, most of wild type cells treated with HU were elongated with one nucleus in the center, which indicates the activation of DRC. In contrast, most of the HU-treated *rad3*Δ cells showed the *cut* phenotype (Fig. 8, red arrows) where the cells were short, formed the septum that separate daughter cells with either undetectable or

different amounts of genomic DNA. The HU-treated *mrc1*Δ cells also elongated but lacked the *cut* phenotype, which is consistent with the functional DDC. The HU-treated *erg11-1* cells clearly showed the septum (Fig. 8, green arrows) and one nucleus in each of the unseparated daughter cells, consistent with the cytokinesis arrest. In the presence of GZ, however, all *S. pombe* cells did not elongate. Furthermore, *rad3*Δ mutant did not show the *cut* phenotype even though the Mrc1 phosphorylation levels were significantly increased. This result suggests that the G2/M arrest caused by GZ prevents *rad3*Δ cells from undergoing a mitotic catastrophe, which provides an additional support to the notion that GZ causes G2/M arrest although the replication stress was generated under the same conditions. Since *rad3*Δ mutants did not show any *cut* phenotype and *erg11-1* cells did not show a cytokinesis arrest, it is possible that GZ stalls the cell cycle at late G2 or M phase.

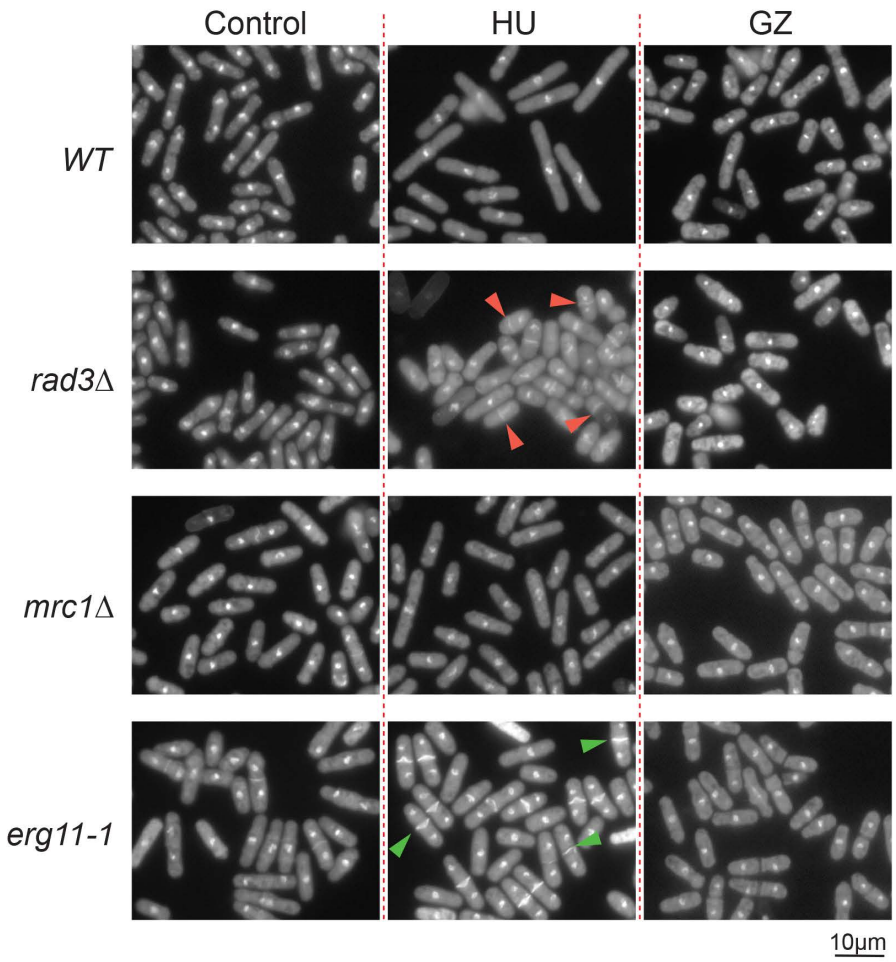

Figure 8: Microscopic examination of wild type, rad3Δ, mrc1Δ, and erg11-1 cells treated with HU or GZ

The cells were treated with 15 mM HU or 200 mM GZ for 3 hours and fixed by brief heating. After staining with Hoechst and Blankophor, they were examined under microscope. The red arrows indicate the *cut* cells in *rad3*Δ induced by HU. The green arrows indicate the cytokinesis arrest in HU-treated *erg11-1* cells.

5. DISSCUSSION

Replication stress is unavoidable in all proliferating cells that leads to DRC activation to prevent DNA damage, mutagenesis, and thus, prevents the formation cancerous cells (52). Although it has been extensively investigated, the mechanism of the DRC is still incompletely understood and need further studies. Hydroxyurea is the commonly used drug in the laboratories to generate replication stress for checkpoint studies. However, it has undesirable side effects that compromise its usage in the checkpoint research. In this study, we tested three other RNR inhibitors GZ, 3-AP, and GEM in *S. pombe* aiming to find a drug that is better than HU in specifically inducing the replication stress. Among these tested agents, GEM did not show any cell growth inhibitory effect in the spot assay at a concentration as high as 77.5 mM. One explanation is that it cannot be transported into the cell due to its bulky structure as compared with HU (Fig. 3). Alternatively, unlike mammalian cells, *S. pombe* lacks the transporter for its influx. In mammalian cells, GEM not only requires a transporting system composed of five nucleoside transporters to enter the cell, but it also requires a nucleoside kinase to be converted into its active metabolites (41,42). *S. pombe* may lack the kinase and the transporters, leading to the insensitivity. It has been reported that expressing the exogenous human equilibrate nucleoside transporter and thymidine kinase from herpes virus increased the incorporation of thymidine analogs into the DNA of fission yeasts (53). It remains to be seen whether GEM is cytotoxic in the *S. pombe* expressing the exogenous transporter and the thymidine kinase. Like GEM,

3-AP is a large and bulky drug. Although it did not cause acute cytotoxicity, it showed a chronic lethal effect in *S. pombe*. The cytotoxicity of 3-AP is thought to be due to replication stress (Fig. 4) because *rad3*Δ and *cds1*Δ cells were significantly more sensitive than the wild type cells. Surprisingly, the fission yeast was insensitive to 3-AP under acute conditions. It is likely due to its bulky structure or its hydrophilicity that hinders it from penetrating the cell wall of *S. pombe* in a short time. Therefore, 3-AP may enter the cell slowly (3 days for chronic vs 8 hours for the acute drug treatment) and show the growth inhibitory effect only in the spot assay.

Among the three tested RNR inhibitors, GZ is the only drug that exhibits both chronic and acute sensitivity in *S. pombe*. In the chronic spot assay, *rad3*Δ and *cds1*Δ mutants are much more sensitive than wild type cells, which indicates the presence of replication stress. Furthermore, GZ is more specific than HU in producing replication stress under chronic conditions since it causes less sensitivity in the metabolic mutants. The *mrc1*Δ mutant was slightly less sensitive to GZ and HU than *cds1*Δ probably due to the basal Rad3-dependent phosphorylation of Cds1 (10). While GZ induces replication stress under acute conditions, it arrests majority of wild type and *rad3*Δ cells in G2/M, not S phase, which strongly suggests a side effect of GZ. This side effect may arise due to the fact that high concentrations of GZ are required to induce replication stress and to activate the checkpoint efficiently. The G2/M cell cycle arrest may function similarly to the activated DDC, which suppress the cytotoxicity caused by the GZ-induced replication stress. Indeed, HU also halts the cell cycle at G2/M in wild type *S.pombe* when 50 mM is

administrated (31). The G2/M arrest induced by higher concentrations of GZ would also explain the reduced cytotoxicity in *rad3*Δ cells (Fig. 4), and the absence of the *cut* phenotype in GZ-treated *S. pombe* (Fig. 8). Together, our results show that GZ and 3-AP can replace HU to produce replication stress more specifically in *S.pombe* only under the chronic conditions such as the spot assay, a method commonly used for genetic studies in yeasts. Between GZ and 3-AP, GZ is a better choice for this assay because of its lower cost. Nevertheless, none of the three RNR inhibitors was able to replace HU for generating acute replication stress in fission yeast. Therefore, using GZ for producing the replication stress under chronic conditions and HU for inducing acute replication stress would complement each other and benefit the checkpoint research. Because of the conserved nature of the DRC throughout the evolution, these results will likely assist the checkpoint research in other model systems.

6. CONCLUSION

DNA is continuously threatened by exogenous or endogenous damaging agents that cause mutations and increase the cancerous risk. However, the cells adapt various control mechanisms that deal with the generated stress to maintain genome integrity and cellular viability. One of these mechanisms is the DNA replication checkpoint which maintains the genome integrity under replication stress. Although it has been investigated for the past decades, the mechanisms of the replication checkpoint remain not fully understood. Hydroxyurea is the commonly used drug in the laboratories to generate replication stress for checkpoint study. Yet, the checkpoint research is sometimes compromised due to the unwanted side effects caused by HU. In this study, we tested three RNR inhibitors GZ, 3-AP, and GEM in *S. pombe* under various experimental conditions in order to find a drug that can produce replication stress more specifically than hydroxyurea. Our data show that among these three drugs, GZ produces replication stress more specifically than hydroxyurea only under chronic conditions. When high concentrations of GZ is used to produce acute replication stress, it arrests the cell cycle at G2/M, not at S phase, indicating a side effect which may indirectly suppress the activation of the replication checkpoint. Together, we propose that employing GZ under chronic conditions and HU in acute experiments can avoid the side effects of both drugs and produce the replication stress more specifically under various experimental conditions. This result will benefit

the checkpoint research in *S. pombe* and likely the related studies in other experimental model systems.

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