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Review article

Hydrogen sulfide and DNA repair

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ABSTRACT

Recent evidence has revealed that exposing cells to exogenous H 2 S or inhibiting cellular H 2 S synthesis can modulate cell cycle checkpoints, DNA damage and repair, and the expression of proteins involved in the maintenance of genomic stability, all suggesting that H 2 S plays an important role in the DNA damage response (DDR). Here we review the role of H 2 S in the DRR and maintenance of genomic stability. Treatment of various cell types with pharmacologic H 2 S donors or cellular H 2 S synthesis inhibitors modulate the G 1 checkpoint, inhibition of DNA synthesis, and cause p21, and p53 induction. Moreover, in some cell models H 2 S exposure induces PARP-1 and g-H2AX foci formation, increases PCNA, CHK2, Ku70, Ku80, and DNA polymerase-d protein expression, and maintains mitochondrial genomic stability. Our group has also revealed that H 2 S bioavailability and the ATR kinase regulate each other with ATR inhibition lowering cellular H 2 S concentrations, whereas intracellular H 2 S concentrations regulate ATR kinase activity via ATR serine 435 phosphorylation. In summary, these findings have many implications for the DDR, for cancer chemotherapy, and fundamental biochemical metabolic pathways involving H 2 S.

1. Introduction

1.1. ATR and the DNA damage response

Maintenance of genomic stability is central for the preservation and perpetuation of life. Nucleic acids are chemically reactive and damaged by a myriad of genotoxic agents. Human cell division necessitates the accurate replication of over six billion base pairs of DNA, while dealing with events such as stalled replication forks, secondary DNA structures, and RNA-DNA hybrid formation. Thus, cells must have efficient and robust DNA replication and repair systems [1]. Central to this process is the DNA damage response (DDR), which detects DNA damage, signals the presence of damage, promotes DNA repair, and maintains the integrity of replicating chromosomes [1,2].

The DDR depends on the activities of three proteins, the DNA-dependent protein kinase (DNA-PK), the *ataxia telangiectasia-mutated* kinase (ATM), and the ATM and Rad3-related (ATR) serine/threonine protein kinase [1,2]. DNA-PK and ATM detect and respond mainly to double-stranded DNA (dsDNA) breaks and cells lacking the expression of either kinase are viable [1–3]. ATR functions in the DDR by stabilizing stalled replication forks, preventing replication stress, initiating cell cycle checkpoints following genotoxic stress, and promoting faithful anaphase chromosomal segregation [1–3]. Homozygous ATR-deficient mouse embryos are inviable and show shattered chromosomes in early embryogenesis [1–3]. Interestingly, hypomorphic ATR mutations are found in the Seckel syndrome type 1, which is characterized by microcephaly, growth retardation, craniofacial abnormalities, intellectual disability, and defects in the DDR [4]. ATR responds to ultraviolet and ionizing radiation, oxidative stress, DNA polymerase inhibition, topoisomerase poisons, dNTP depletion, stalled replication forks, and DNA cross-linking and alkylating agents [1–3,5]. Many of these genotoxic stressors share the common structural component of single-stranded DNA (ssDNA) [3,5].

ATR activation is initiated when replication protein A (RPA) binds ssDNA at a stalled replication fork, forming a platform that recruits multiple proteins, including several ATR activating proteins and ATR itself, leading to phosphorylation of CHK1 by ATR, followed by subsequent CHK1 kinase activation [3]. Following complex formation,

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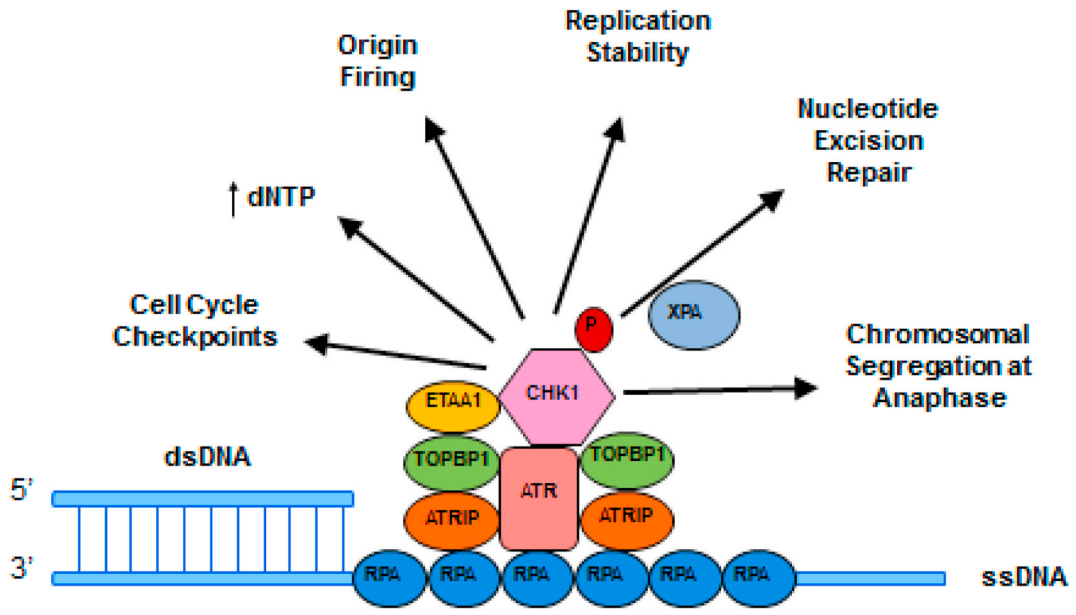

Fig. 1. A schematic depiction of ATR activation. Replication protein A (RPA) binds ssDNA allowing the binding of ATR-interacting protein (ATRIP) and topoisomerase II binding protein (TOPBP1), permitting ATR to bind to the complex. RPA also binds Ewing tumor-associated antigen 1 (ETAA1), assisting in ATR activation. Activated ATR phosphorylates CHK1 serine 345, activating the kinase and subsequent downstream signalling events. Upon ultraviolets radiation exposure ATR is phosphorylated on serine 435 allowing xeroderma pigmentosum complementation group A (XPA) protein binding and subsequent nucleotide excision repair.

ATR/CHK1 initiates cell cycle checkpoint arrest, stabilizes stalled replication forks, regulates DNA replication origin firing, preventing excessive origin firing and suppressing firing in response to replicative stress, and also increases ribonucleotide-diphosphate reductase subunit M2 activity, increasing dNTP production [3,5]. In the case of UV-mediated DNA photodamage, ATR serine 435 is phosphorylated, allowing the recruitment of xeroderma pigmentosum complementation group A (XPA) protein, an event necessary for nucleotide excision repair [3,5]. These events are summarized in Fig. 1.

1.2. Hydrogen sulfide and DNA repair

Hydrogen sulfide (H_2S) is a recently identified endogenously synthesized gasotransmitter with an ancient biochemistry that likely played an essential role in the emergence of life [6,7]. H_2S functions in many different organ and biochemical systems, including the digestive, cardiovascular, renal, central nervous, respiratory, and antioxidant defense systems [6]. H_2S is primarily synthesized by three enzymes: cystathione β-synthase (CBS), cystathione γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST), with each enzyme exhibiting different substrate specificities, and intracellular and organ distributions [6].

Interestingly, H_2S has been shown to modulate DNA stability, exerting different effects at different concentrations and exposure times. For example, nuclei from Chinese hamster ovary cells treated for 2 h with as little as 1 μM Na_2S showed significant DNA damage, an event reversed by treatment with butyl-hydroxyanisole [8]. Similarly, H_2S treatment of human lung fibroblasts with as low as 10 μM NaHS for 12 h caused micronuclei formation, and induced p53, p21, Bax, cytochrome *c*, Ku-70, and Ku-80 protein expression, with a concomitant G_1 cell cycle arrest. Bax also translocated from the cytosol to the mitochondria, with cytochrome *c* being release from the mitochondria [9]. This latter finding potentially implicates H_2S in the DDR, as the Ku-70/Ku-80 heterodimer associates with DNA-PK, to promote non-homologous end joining to repair dsDNA breaks [1]. Similarly, two glioblastoma cell lines treated with 476 μM Na_2S for 4 h, showed increased DNA damage, cellular oxidative stress, and significantly increased γ-H2AX foci formation [10]. This later observation is interesting as H2AX phosphorylation to form γ-H2AX requires ATM, ATR, and DNA-PK kinase activities, further implicating the DDR in H_2S biology [1,2,11]. In human small intestinal epithelial cells 500 μM Na_2S at 0.5 and 4 h exposures induced significant genotoxic, but not cytotoxic damage, and gene array analyses at both time points revealed the induction of genes related to DNA metabolism and repair, cell cycle arrest, and the regulation of apoptosis [12].

H_2S also induces cell damage responses at relatively lower concentrations [13–15]. For example, treatment with 5 ng/ml H_2S (~140 nM) for 24 h significantly decreased DNA synthesis, induced a G_1 cell cycle checkpoint, reduced retinoblastoma protein phosphorylation, and induced p21 protein expression in the Ca9-22 gingival squamous cell carcinoma cell line [13]. Similarly, human gingival fibroblasts cultured 72 h with 100 ng/ml H_2S showed a significant increase in apoptosis, caspase 3 activity, and reactive oxygen species levels, with a concomitant suppression of superoxide dismutase activity [14]. Lastly, treatment of oral keratinocyte stem cells with 50 ng/ml H_2S for 24 and 48 h significantly increased cellular apoptosis, caspase 3 and 9 activities, serine 46 p53 phosphorylation, CHK2, PTEN, Bcl-2, and SIRT3 protein levels, and induced growth arrest [15]. Again, in relation to the DDR, this response is interesting as p53 serine 46 phosphorylation involves ATM activity [16].

Conversely, other studies have found that H_2S and its reactive sulfur derivatives can enhance genomic stability. For example, daily intraperitoneal injection of the H_2S donor diallyl sulfide at 50 mg/kg into female rats, induced p53, Gadd45a, PCNA, and DNA polymerase delta in their breast tissue, suggesting that H_2S enhances breast tissue DNA repair capacity [17]. Treatment of human endothelial cells with as little as 1 μM NaHS resulted in MEK1 S-sulfhydration at cysteine 314, facilitating the translocation of phosphorylated ERK1/2 in the nucleus, initiating significant PARP-1 activation with subsequent XRCC1 and DNA ligase III recruitment to DNA breaks, enhancing DNA repair and protecting the cells from senescence [18]. Similarly, compared to normal lung epithelial cells, cultured lung adenocarcinoma cells have elevated H_2S-synthseizing enzymes and H_2S levels, and increased EXOG sulfhydration, which promotes mitochondrial DNA repair complex assembly, and mitochondrial DNA repair capacity [19]. Inhibition of H_2S synthesis with 300 μM aminooxyacetic acid (AOAA, a CBS/CSE inhibitor) in adenocarcinoma cells suppressed mitochondrial bioenergetics

and increased cellular sensitivity to the chemotherapeutic agent camptothecin [19]. Correspondingly, in murine smooth muscle and aorta tissue, CSE deficient cells show reduced mitochondrial DNA copy numbers, mitochondrial content, mitochondrial-specific mRNAs (*MT-CO1, CytB*, and *Atp 6*), and mitochondrial transcription factor A mRNA and protein expression (TFAM), compared to syngeneic CSE wild type cells. Treatment with 30 and 60 μM NaSH for 48 h significantly increased the expression of all these mitochondrial markers [20]. The CSE deficient cells also showed elevated DNA methyltransferase 3A (Dnmt3a) expression, accompanied by increased global DNA methylation and increased methylation of the TFAM promoter [20]. Treatment with 30 μM NaSH suppressed Dnmt3A expression in the CSE deficient cells and TFAM promoter methylation, increasing TFAM protein expression [20]. Thus CSE-derived H_2S plays an important role in maintaining the mitochondrial genome, regulating global gene methylation, and specific gene expression. Last, in different studies H_2S has been shown to stimulate cell proliferation and prevent apoptosis, while in other studies it suppresses cell growth, lowering cell viability, and increasing apoptosis [21].

Together, these studies demonstrate that H_2S can induce DNA damage, cell checkpoint responses, increase the expression of proteins associated with cell cycle checkpoints and the DDR, and modulate mitochondrial and nuclear genomic stability. Based on this, our group has examined the role of H_2S in regulation of the DDR, with a specific focus on the ATR kinase [22].

1.3. ATR, H_2S, and the DDR

Our analysis involved comparing two syngeneic cell lines, one carrying wild type ATR (ATR cells) and the other carrying biallelic knock-in hypomorphic ATR Seckel syndrome I genes (ATR-H cells) [22,23]. Colony forming efficiency assays were utilized to determine if the ATR-H cells showed defects in cell growth responses following exposures to either an H_2S synthesis inhibitor (β-cyano-L-alanine), the H_2S donor diallyl trisulfide, or no pretreatment, following *t*-butyl-hydroperoxide (*t*-BOOH) treatment [22]. ATR-H cells exhibited an increased sensitivity to the same *t*-BOOH doses, compared to the ATR cells. Pretreatment with the H_2S synthesis inhibitor or donor increased cellular sensitivity to *t*-BOOH in both cell types, with the ATR-H cells showing greater sensitivity to all treatments combinations, compared to the ATR cells [22]. Based on this, we hypothesized that ATR may play a role in H_2S metabolism.

To further examine this hypothesis, we directly measured intracellular H_2S concentrations using high performance liquid chromatography (HPLC) analysis of sulfide dibimane, a specific reaction product of H_2S with monobromobimane [24]. ATR-H cells had significantly lower H_2S concentrations than ATR cells, and interestingly, treatment with the ATR kinase inhibitor NU61027 significantly lowered H_2S concentrations in the wild type ATR cells, but not ATR-H cells [22]. Additionally, the CBS/CSE inhibitor β-cyano-L-alanine further suppressed H_2S concentrations in both cell types, indicating that H_2S intracellular concentrations are regulated at two distinct molecular nodes; 1) by ATR kinase activity, and 2) by H_2S synthesis involving CBS and CSE [22]. Lastly, we used western blotting to compare CBS, CSE, and 3-MST protein expression in the ATR and ATR-H cells. All three of these enzymes were significantly lower in the ATR-H cells compared to the ATR cells [22].

ATR activation correlates with ATR serine 435 phosphorylation, an event required for ATR-XPA complex formation, which promotes nucleotide excision repair at sites of photodamaged DNA [5]. When we treated ATR cells with the H_2S synthesis inhibitor β-cyano-L-alanine, Western blot analyses revealed that ATR serine 435 phosphorylation was significantly increased compared to untreated ATR cells, while treatment with the H_2S donor diallyl trisulfide significantly suppressed this phosphorylation. As previously found, treatment of the two cell types with either *t*-BOOH or UV exposures, increased ATR 435 phosphorylation in the ATR cells, but not in the ATR-H cells [5,22]. These treatments did not alter ATR serine 435 phosphorylation in the ATR-H cells [22]. This data revealed an important regulatory feedback loop involving ATR kinase phosphorylation by intracellular H_2S concentrations [22]. The data further implies, but does not directly show, that nucleotide excision repair may also be modulated by intracellular H_2S levels [5,22].

Since H_2S synthesis inhibition increases the serine 435 phosphorylation associated with ATR kinase activation, our data implied that H_2S synthesis inhibition could significantly alter ATR kinase activity [5]. Following its activation, ATR phosphorylates the CHK1 kinase on serine-345, an event resulting in CHK1 kinase activation [2,25]. Pretreatment of ATR cells with β-cyano-L-alanine, followed by a short treatment with a low *t*-BOOH concentration, resulted in higher CHK1 serine-345 phosphorylation than the same *t*-BOOH concentration without β-cyano-L-alanine pretreatment [22]. Thus, pharmacologic lowering of intracellular H_2S concentrations potentiates ATR kinase activity following oxidative stress. As with previously performed experiments, these results were not found in the ATR-H cells [22]. Lastly, as β-cyano-L-alanine, an inhibitor of CBS and CSE, alters both ATR phosphorylation and kinase activity, the data shows that these enzymes activities play a role in modifying the DDR.

Finally, to confirm that the interactions of the ATR kinase and H_2S play a direct role in genomic stability, we directly measured double-stranded DNA (dsDNA) breaks in ATR and ATR-H cells by oil emersion microscopy. This technique was chosen as many assays that measure DNA damage depend directly on the correct functioning of the DDR proteins, something partially ablated in the ATR-H cells [4,22,23]. ATR and ATR-H cells were pretreated with β-cyano-L-alanine followed by treatment with a low concentration of *t*-BOOH or treated the same concentration of *t*-BOOH without the pretreatment. The colcemid metaphase-blocked Giemsa stained chromosomal preparations showed a non-significant increase in dsDNA breaks following exposure to a low *t*-BOOH concentration in both cell types. However, pretreatment with β-cyano-L-alanine followed by the same *t*-BOOH concentration resulted in a significant increase in dsDNA breaks in both cells types [22]. Thus, low-level oxidative stress that does not cause significant increases in dsDNA breaks alone, does cause significant breaks when H_2S synthesis is inhibited, demonstrating that H_2S plays an important role in the maintenance of genomic stability.

1.4. Experimental H_2S donor and H_2S synthesis inhibitor use

An important and sometimes unaddressed aspect of these studies, and many studies on H_2S, is the use of H_2S donors and H_2S synthesizing enzyme inhibitors. H_2S concentrations within cells are rather low, less than 1 μM in most studies [24]. Additionally, a recent comparison of H_2S concentrations in quick-frozen and analyzed biopsies of oral squamous cell carcinomas compared to benign adjacent oral epithelium, revealed a statistically significant, but smaller (~13%) increase in free H_2S concentrations in the malignant tissues [26]. Thus, even with malignant transformation, cellular H_2S levels are likely only moderately increased. However, many studies have treated cultured cells with supraphysiologic H_2S donor concentrations, raising the possibility that such experimental findings represent non-physiologic/non-pathophysiologic cellular responses. For example, 25, 75, and 476 μM Na_2S and 10 μM NaHS were used to treat different cell cultures, examining radical-associated DNA damage, apoptotic cellular responses, mitochondrial dysfunction, and PARP-1 activation (8–10,18). Additionally, Na_2S and NaHS are rapid H_2S-releasing drugs which instantaneously disassociate in solution, releasing a high and extremely rapid H_2S dose which is biochemically very unlike the much slower, steady-state H_2S production by the three H_2S-synthesizing enzymes [27]. Thus, it is likely that even lower dosages of these H_2S donors deliver rapid and possibly supraphysiologic H_2S dosages [27]. Free H_2S also exists in a rapid equilibrium with higher order polysulfides, many of which likely exert unique biological effects [28]. Thus, high and rapid H_2S doses likely

involve currently poorly defined biological effects secondary to higher order polysulfur compound formation, conceivably adding biological effects not seen at more physiologic H_2S concentrations [28].

Taken together, these concerns raise the question that much of the data on H_2S donors, especially the rapidly disassociating ones, may be non-physiologic/non-pathophysiologic and therefore require careful consideration and where possible, should be repeated with slower H_2S-releasing donors, such as GYY4137, preferably at low μM or even nM concentrations [27]. It is important that cellular events induced by supraphysiological H_2S concentrations be separated from those relevant to normal physiology versus pathophysiologic events. To some degree this has been done, as in several of the studies cited above, biologically significant effects were identified at 1 μM and 10 μM H_2S donor concentrations, which are high physiologic to pharmacologic H_2S concentrations [8,10,18,24]. Also, two studies employing gingival epithelial cells revealed low 5–50 ng/ml H_2S concentration induce cell cycle checkpoint arrest, apoptosis, and modulation some genes involved in events downstream of the DDR. These effects were seen with a longer 24-h treatment time and more likely resemble physiologic H_2S concentrations [13,15]. Lastly, H_2S concentrations in the intestines can be quite high (~1 mM), thus for studies done on intestinal epithelial cells, high H_2S donor concentrations may be appropriate [29].

Interestingly, in two studies 50 and 200 μM NaHS restored a normal morphologic phenotype to Werner syndrome fibroblasts and rescued rat mesenchymal stem cells from hypoxia-induced cell apoptosis, respectively [30,31]. In the Werner syndrome fibroblasts, the cells were treated for one week with 50 μM NaHS daily and harvested. NaHS treatment abrogated Werner cell protein aggregation, decreased mTOR protein expression and mTOR serine 2448 phosphorylation, and increased Akt protein phosphorylation. Interestingly, CBS and CSE protein expression were lower Werner syndrome fibroblasts, compared to wild type fibroblasts [30]. Treating male rat mesenchymal bone marrow stem cells with 200 μM NaHS reduced hypoxia-induced cell apoptosis and increased stem cell survival when translated into female rat's hearts immediately after myocardial infarction induction. Stem cell NaHS treatment reduced the infarct size and increased left ventricular function [31]. These changes were accompanied by increased phosphorylated Akt, Erk1/2, and glycogen sythase-3β [31]. Thus, cellular exposures to sudden and high H_2S concentrations can in some cases exert beneficial biological effects, suggesting that these H_2S doses, although possibly non-physiologic, are not always deleterious to cellular function.

Many studies employ H_2S-synthesizing enzyme inhibitors to lower cellular H_2S concentrations. The currently used H_2S-synthesizing enzyme inhibitors are not entirely selective and may inhibit other cellular pathways. For example, the commonly used CBS inhibitor AOAA inhibits CSE and over thirty other cellular enzymes, greatly complicating the analysis of experiments using this compound [32]. In our work on H_2S and ATR, we used β-cyano-alanine, a stronger CSE inhibitor and a weaker CBS inhibitor, which also inhibits β-decarboxylase and alanine aminotransferase [22,33]. Additionally, inhibition of the H_2S-synthesizing enzymes changes the concentrations of the transsulfuration pathway precursors and products. For example, CBS inhibition results in lower H_2S synthesis and homocysteine accumulation combined with a concomitant lowering of cytoprotective cystathione and glutathione concentrations [32]. Obviously, many of the cellular responses attributed to H_2S-synthesizing enzyme inhibition may also be in part due to changes in these other metabolite concentrations and increased cellular oxidative stress. The current lack of both potent and specific H_2S-synthesizing enzyme inhibitors continues to be a limitation in this field making analyses of data when using these compounds challenging.

In our work on H_2S and the ATR kinase we found that by itself, 50 μM of the H_2S donor diallyl trisulfide inhibited growth of the colon cancer cell line DLD1 about 75% in the colony forming assay compared to untreated cells [22]. We therefore used a lower 20 μM concentration in our analyses of ATR phosphorylation and the effects of diallyl trisulfide on intracellular H_2S concentrations [22]. Based on these results, the diallyl trisulfide concentration we used is likely close to a physiologic H_2S dose. Future studies in this area should examine the effects of H_2S on ATR-mediated events, such as ATR-dependent cell cycle checkpoints, DNA replication regulation, the stabilization of stalled replication forks, and ATR-mediated signal transduction pathways, such as those stemming for the CHK1 and other ATR-regulated kinase activities.

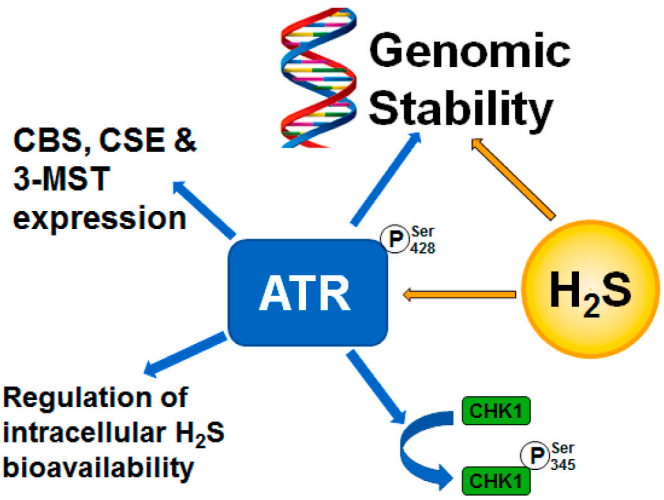

Fig. 2. A summary of the interactions of H_2S and the ATR kinase. ATR regulates intracellular H_2S and the expression of the H_2S synthesis enzymes. H_2S in turn, regulates ATR serine 435 phosphorylation and ATR kinase activity towards the CHK1 kinase. Last, both ATR and H_2S function together to promote genomic stability.

2. Conclusion

The ATR kinase and H_2S reciprocally regulate each other, summarized in Fig. 2. These data have several implications. First, in many human cancers, H_2S synthesis is increased and H_2S synthesis inhibition decreases tumor growth, tumor angiogenesis, metastatic potential, and increases tumor sensitivity to chemotherapeutic agents [6,19,26,34–36]. Thus, tumor H_2S synthesis inhibition has been proposed as a cancer treatment [6]. Similarly, ATR inhibition has also been proposed as a cancer treatment and the ATR pharmacologic inhibitor we examined in our studies (NU61027) is now being used in clinical trails as a cancer chemotherapeutic agent [37]. Based on this, tumor cell ATR inhibition may both lower tumor cell DNA repair capacity and suppress tumor H_2S synthesis, thus simultaneously "hitting" two cancer chemotherapeutic targets. Such a cancer chemotherapeutic regime may have greater efficacy for cancer treatment. Additionally, as H_2S synthesis is controlled by both ATR activity and CBS/CSE activities, cancer chemotherapeutic regimes that simultaneously target both these foci of H_2S synthesis may synergetically suppress tumor cell H_2S levels, resulting in an enhanced chemotherapeutic effect. Some support for this idea comes from the observation that several common malignant tumors show increased CBS and CSE expression, suggesting that such a chemotherapeutic modality may preferentially inhibit H_2S-dependent pathways required for tumor cell functioning [6,19,26,34–36]. Additionally, tumor cells often show widespread DDR activation with concomitant increased and chronic replicative stress [37–41]. Thus, like H_2S synthesis inhibition, ATR inhibition may also preferentially inhibit tumor cell growth and survival. This concept is summarized in Fig. 3.

In summary, current studies demonstrate that H_2S plays an important role in regulating DNA stability and repair [8–10,12–22]. Our data links on H_2S to ATR functions and the DDR and implies that H_2S may regulate nucleotide excision repair, chromosomal segregation at

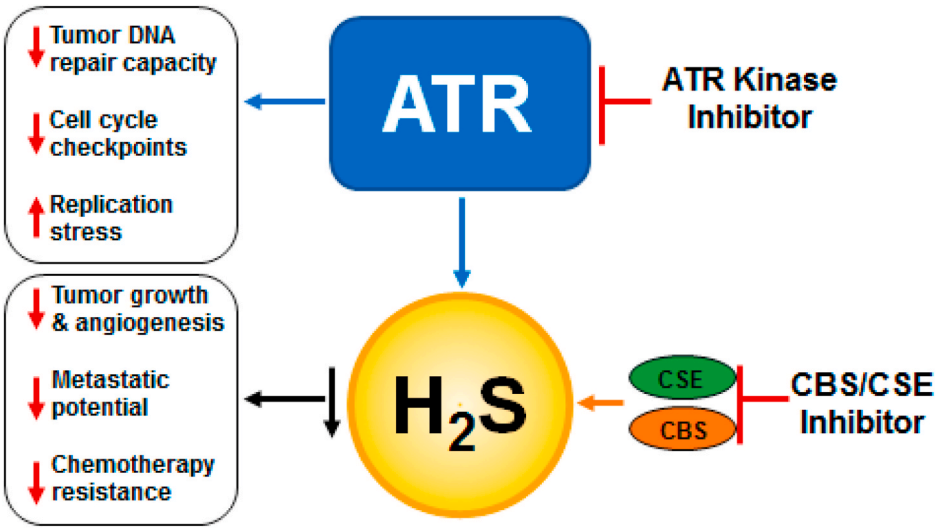

Fig. 3. A summary of a cancer chemotherapeutic regimen that would inhibit H_2S synthesis at two molecular foci of H_2S synthesis, possibly resulting more effective tumor chemotherapy treatment.

anaphase, the stabilization of replication S-phase fork integrity, and other ATR kinase dependent activities [1–3,22]. Similarly, possible interactions between the ATM and DNA-dependent protein kinases and H_2S should also be examined, as these proteins have significant functional overlap and sequence homology to ATR, suggesting that they might also interact with H_2S [1–3,5].

Declaration of competing interest

The author's report no conflict of interest.

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Appendix A. Supplementary data

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