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13. ABSTRACT *(Maximum 200 Words)*
Adozelesin is an alkylating minor groove DNA binder that is capable of rapidly inhibiting DNA replication in treated cells through a trans-acting mechanism. It has been shown previously that in cells treated with adozelesin, replication protein A (RPA) activity is deficient and the middle subunit of RPA is hyper-phosphorylated. The adozelesin-induced RPA hyper-phosphorylation can be blocked by the replicative DNA polymerase inhibitor aphidicolin, suggesting that adozelesin-triggered cellular DNA damage responses require active DNA replication forks. This indicated that adozelesin induces S phase-specific DNA damage responses. Here we show that RPA hyper-phosphorylation, RPA intra-nuclear focalization, and γ-H2AX intra-nuclear focalization induced by adozelesin treatment are all dependent on DNA replication fork progression, and the foci are only induced in S phase cells. These findings are the same as those seen with the S phase specific DNA damaging agent, camptothecin. Conversely, all three DNA damage responses are independent of either S phase or replication fork progression when induced by treatment with the DNA strand scission agent, C-1027. Further, we demonstrate that adozelesin-induced RPA and γ-H2AX intra-nuclear foci co-localize within the nuclei of S phase cells.

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Introduction:

In this report, we study the cellular mechanism of adozelesin-induced DNA replication arrest. Adozelesin is an analog of CC-1065, a cyclopropylpyrroloindole (CPI) isolated from Streptomyces zelensis. Several CPI compounds have entered clinical studies for solid tumors, including breast cancer. Adozelesin is capable of binding to the minor groove of A/T-rich DNA sequences and alkylating the N3 of adenine at 3'-end of the binding sites (1, 21). These two activities contribute to its anti-cancer ability. Binding of adozelesin does not distort the duplex structure of targeted DNA (1) or cause any DNA strand break (2). Although nucleotide excision repair might be involved in the removal of CPI-induced lesions (4, 6), CC-1065:DNA adducts persist in BSC-1 green monkey cells (22). It is possible that these DNA adducts are been repaired inefficiently in treated cells.

Previously published results showed that adozelesin inhibits both cellular and viral DNA replication in treated cells through a trans-acting mechanism. The trans-acting replication factor that is inactivated upon the action of adozelesin has been identified as replication protein A (RPA) (8). RPA is the major eukaryotic single-strand DNA (ssDNA) binding protein. This heterotrimeric protein (70, 32 and 14 kDa) is essential for DNA replication, and plays critical roles in DNA repair and recombination (5, 19). During S phase of the cell cycle or when cells are subjected to DNA damage, a portion of RPA becomes tightly associated with the nuclear matrix to form intranuclear foci, and the 32-kDa subunit of RPA (RPA32) is hyper-phosphorylated (5, 19).

Our published results showed that adozelesin-induced RPA32 hyper-phosphorylation could be prevented by pre-treating the cells with DNA polymerase inhibitor aphidicolin (8). These findings suggested that RPA32 hyper-phosphorylation induced in cells treated with adozelesin requires DNA replication fork progression. Reports from other groups demonstrated the similar requirement of replication fork passage for RPA32 hyper-phosphorylation induced by camptothecin or UV radiation (13, 15, 17). However, in cells treated with enediyne C-1027, a DNA scission agent that directly binds and breaks one or both strands of DNA (7, 12, 20), induction of RPA32 hyper-phosphorylation was completely resistant to aphidicolin (12), suggesting a mechanism that is independent of DNA replication. It is known that camptothecin selectively induces S phase DNA damage checkpoints and causes double-strand DNA breaks (DSBs) only when there is DNA replication fork progression (18). It is obvious how replication forks collide with camptothecin-arrested topoisomerase adducts could result in DSBs that then trigger RPA32 hyper-phosphorylation by DSB-induced kinases. However, for treatments that damage DNA directly, such as adozelesin or UV, why replication fork progression would be required for RPA32 hyper-phosphorylation remains unclear. It has not been reported whether DNA damage-induced RPA focalization also depends on DNA replication fork progression.

In order to further understand the action of adozelesin, two other DNA damaging agents camptothecin and C-1027 were also used in this study as comparison. Camptothecin as stated above has long been known to selectively induce S phase DNA damage checkpoints (for review, see (10)). In contract, DNA scission agent C-1027 causes DNA strand breaks in all the treated cells, therefore induces DNA damage responses independent of cell cycle. The DNA damage markers used to monitor the drug effects were phosphorylated histone H2AX at serine 139 (γ-H2AX), RPA and Chk1 kinase. Induction of γ-H2AX foci is known as an early cellular response to either DSBs or replicational stress (for review, see (14). Since the kinases responsible for DNA damage-induced phosphorylation of H2AX, Chk1 and RPA32 are members in the PI3

kinase family, we used cell lines that are deficient in the function of these kinases to study the possible linkage of phosphorylation and focus formation.

Body

Adozelesin induces S phase-specific DNA damage responses. As shown in Fig. 1, both camptothecin and adozelesin induced RPA and γ-H2AX focus formation in ~40% of cells which were sensitive to aphidicolin pre-treatment. Conversely, C-1027 induced RPA and γ-H2AX focus formation in over 90% of cells. Suggesting that both camptothecin and adozelesin trigger DNA damage responses in S phase cells. Pulse labeling of cells with bromouridine deoxyribonucleotide (BUdR) indicated that camptothecin and adozelesin-induced RPA foci indeed appeared only in S phase cells (Fig. 2). The details of this project have been submitted for publication.

Based on results stated above, in conjunction with previously published reports, we propose a model (Fig. 3) for how various types of DNA damage are affected by replication fork progression. It is clear that DNA scission agent C-1027 is capable of inducing cellular DNA damage responses directly. Replication fork movement is not required for the induction of either RPA or γ-H2AX focus formation. For indirect damage on DNA (such as camptothecin) or damage that is poorly recognized by cellular DNA repair pathways (such as adozelesin), checkpoint mechanisms are activated only when replication forks collide with these adducts. While camptothecin treatment can induce DSBs in cells with active DNA replication fork progression, alkylation of DNA by adozelesin produces no detectable DNA strand break (Fig. 7C) (2). Adozelesin can bind and alkylate DNA in spite of replication fork progression. The absence of RPA and γ-H2AX foci suggests that adozelesin-induced DNA adducts are intractable to DNA repair enzymes, until the collision of replication forks and drug:DNA adducts results in stalled replication forks. Stalled replication forks have been shown to be able to induce S phase checkpoint responses (3, 16). We therefore believe that adozelesin-induced S phase-specific checkpoint responses are triggered by stalled replication forks rather than DNA damage itself.

Adozelesin-induced RPA focus formation and RPA32 hyper-phosphorylation are separable. To further study adozelesin-induced RPA focus formation and RPA32 hyper-phosphorylation, monolayer cultured HeLa cells were treated with adozelesin for various length of time. Focus formation of γ-H2AX was used as a timing marker. Adozelesin-induced γ-H2AX foci can be seen within 10 min of the treatment (Fig. 4). Consider the length of time for drugs to enter the cells and damage DNA, γ-H2AX focus formation must be the immediate response to the damage. The signal of γ-H2AX reached plateau at about 20 min. RPA foci in treated cells were not detectable until 20 min and reach maximal intensity at between 40-60 min, slower than γ-H2AX focus formation (Fig. 4).

As for adozelesin-induced checkpoint kinase activity, both H2AX and Chk1 were phosphorylated within 20 min of treatment (Fig. 5). To our surprise, adozelesin-induced RPA32 hyper-phosphorylation was not detected until 90 min and reached plateau at around 150-180 min (Fig. 5), which was much slower than adozelesin induced RPA focus formation (Fig. 4). These results suggested that although both RPA focus formation and RPA32 hyper-phosphorylation can be induced by DNA damage, these two mechanisms are separable. Our data also suggests that DNA damage checkpoint kinases responsible for histone H2AX and Chk1 phosphorylation and Chk1 activation are turned on immediately after the DNA damage. As for RPA

phosphorylation, either a secondary kinase is responsible for this modification or additional factor is needed.

To identify the possible checkpoint kinases involved in the regulation of RPA focus formation and RPA32 hyper-phosphorylation, mutant or transfected cell lines with functional defect in the members of PI3 kinases (DNA-PK, ATM or ATR) were treated with adozelesin. Adozelesin was able to induce RPA32 hyper-phosphorylation in ATM$^{(-/-)}$ cells and cells transfected with dominant ATR mutant (ATR-kd), but reached plateau at much later time points (4-6 hr) (Fig. 6A and data not shown). The delayed appearance of RPA32 hyper-phosphorylation was not found in DNA-PK$^{(-/-)}$ cells (Fig. 6B). However, according to its migration in SDS-PAGE, RPA32 was phosphorylated instead of hyper-phosphorylated in DNA-PK$^{(-/-)}$ cells treated with adozelesin.

For adozelesin-induced RPA focus formation in these PI3 kinase deficient cell lines, the timing and intensity were indistinguishable to normal cells (Fig. 7) (9). These results suggested that RPA is capable of sensing adozelesin-induced stalled replication forks without rely on any of the known S phase checkpoint kinases.

Key Research Accomplishments:

Tasks 1-3 were finished and the results had been published (8, 9, 12).

Task 4 was finished. The results showed that RPA purified from cells treated with adozelesin retains its single-stranded DNA-binding activity but is selectively inactivated as a DNA replication factor. Its function in nucleotide excision repair (NER) is not affected. Manuscript in preparation.

Task 5-7 are just started. One of the trans-acting inhibitors was identified as DNA-PK (9). We are trying to purify enough DNA-PK protein from suspension cultured 293 cells to test its effect in cell-free SV40 DNA replication assays.

Task 8 is partially presented in this report. Adozelesin-induced DNA adducts are possibly removed by NER pathway. We have set up the cell-free NER assay that is able to repair UV-damaged DNA templates. We will generate adozelesin-damaged plasmids in vitro and test whether they can be repaired in the cell-free NER reactions.

Reportable Outcomes:

(1) Manuscript:

Mary M. McHugh, Xia Yin, Shu-Ru Kuo, Jen-Sing Liu, Thomas Melendy, and Terry A. Beerman. (2001). The cellular response to DNA damage induced by the enediynes C-1027 and neocarzinostatin includes hyperphosphorylation and increased nuclear retention of Replication Protein A (RPA) and trans inhibition of DNA replication. Biochemistry 40:4792-4799.

Jen-Sing Liu*, Shu-Ru Kuo*, Xia Yin, Terry A. Beerman, and Thomas Melendy. (2001). DNA damage by the enediyne C-1027 results in the inhibition of DNA replication by loss of replication protein A function and activation of DNA-dependent protein kinase. Biochemistry 40:14661-14668.

Jen-Sing Liu*, Shu-Ru Kuo*, Terry A. Beerman and Thomas Melendy. Induction of DNA damage responses by adozelesin is S phase-specific and dependent on active replication forks. Submitted.

Jen-Sing Liu*, Shu-Ru Kuo*, Terry A. Beerman, and Thomas Melendy. Different mechanisms and functions of DNA damage-induced intra-nuclear focalization and hyper-phosphorylation of replication protein A. *(in preparation).*
(*: Authors with equal contribution)

(2) Meeting Abstract:

Jen-Sing Liu, Shu-Ru Kuo, Terry A. Beerman, and Thomas Melendy.
DNA replication-dependent and -independent mechanisms for DNA damage-induced intra-nuclear redistribution and hyper-phosphorylation of RPA.
"Eukaryotic DNA Replication", Cold Spring Harbor Laboratory. Sept. 5-9, 2001.

(3) Grant Application:

"Project III: Response of replication and repair factors to DNA damage."
PI: Dr. Thomas Melendy (Mentor of this Fellowship)
Other PIs: Drs. Terry A. Beerman (Co-mentor) and David Kowalski
P01 (NIH/NCI), Submitted (site visit date: May 15, 2002)

Conclusions:

(1) In this report, we have demonstrated that adozelesin induces S phase-specific DNA damage checkpoint responses in treated cells. Considering the high growth rate of tumor cells, chemotherapeutic agents that target on either S or M phase of cell cycle certainly will have higher impact on malignant tissues. In cultured cells, concentration of adozelesin is 2 to 3 order lower than that required for camptothecin to trigger similar cellular responses, suggests that this family of alkylating minor groove DNA binders have high potential to be efficient anti-tumor agents.

(2) RPA focus formation and RPA32 hyper-phosphorylation have been used as markers for S phase DNA damage checkpoint responses. Genetic studies in the yeast suggested that RPA is functioned as an S phase checkpoint factor (11). Based on the time course experiments (Figs. 4 & 5), we believe that RPA focus formation is possibly associated with RPA's checkpoint function, but RPA32 hyper-phosphorylation is not. Since adozelesin-induced RPA focus formation was slower than that of γ-H2AX, we suspect that there is an upstream factor required for RPA focalization. We will continue to find the possible mechanism that connects adozelesin-induced replication fork blockage and RPA focus formation.

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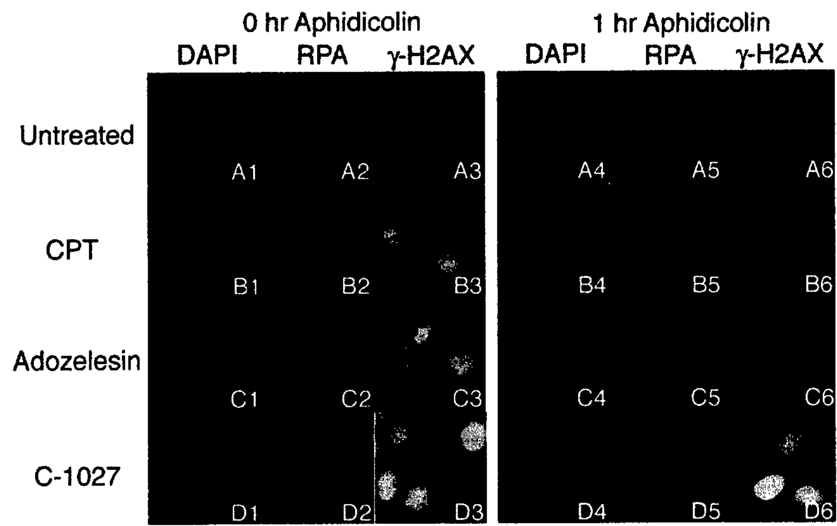


Figure 1

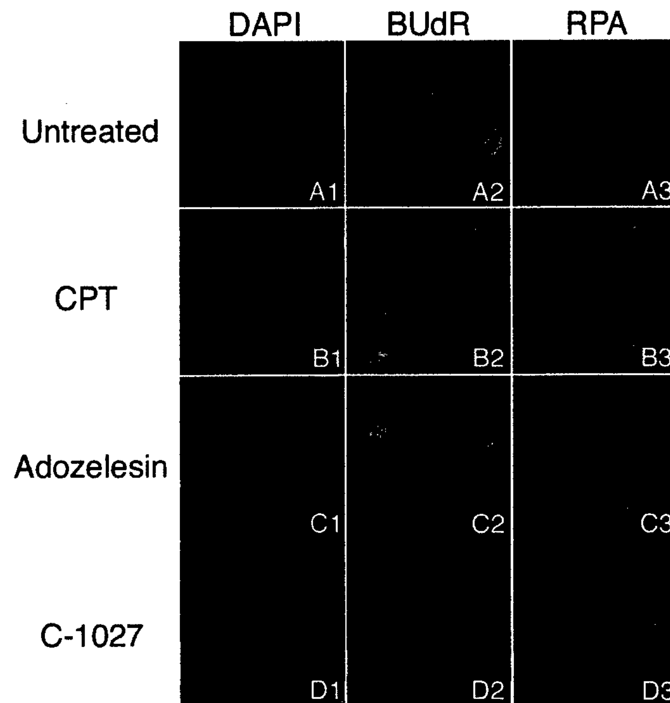
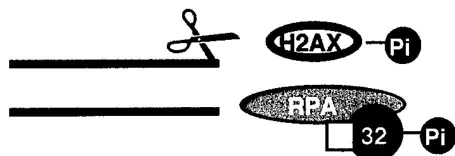


Figure 2

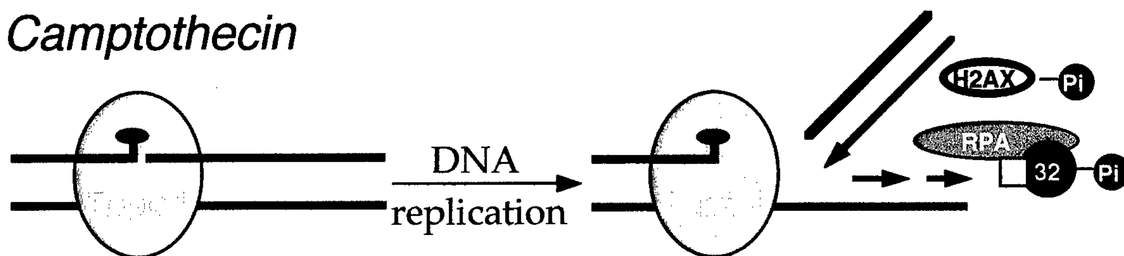
(A) Double Strand Break

C-1027



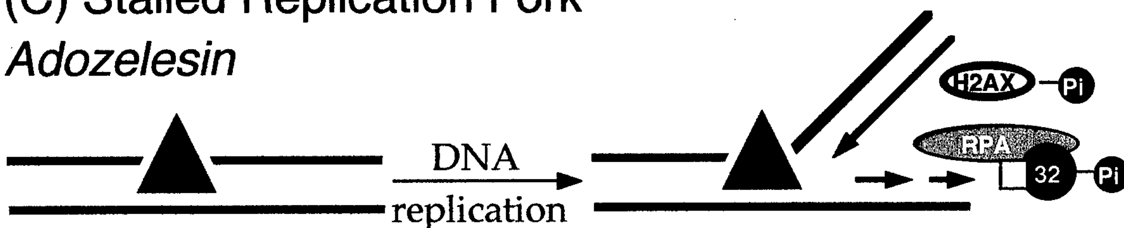
(B) Stalled Replication Fork + Double Strand Break

Camptothecin



(C) Stalled Replication Fork

Adozelesin



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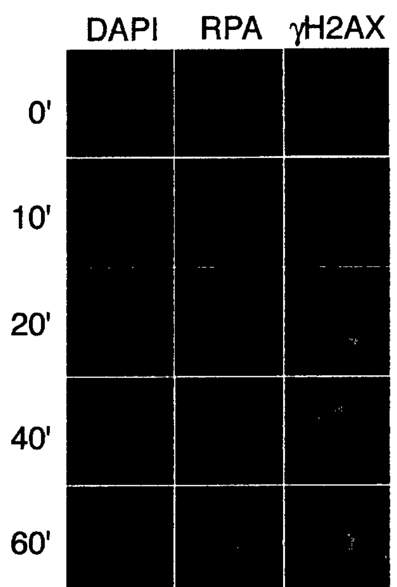

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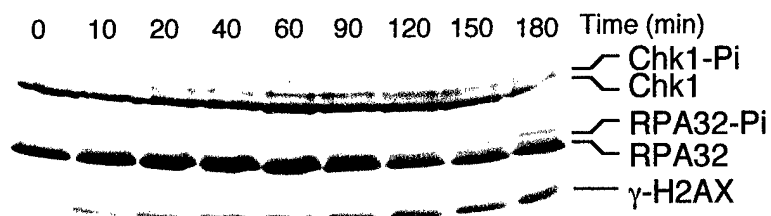

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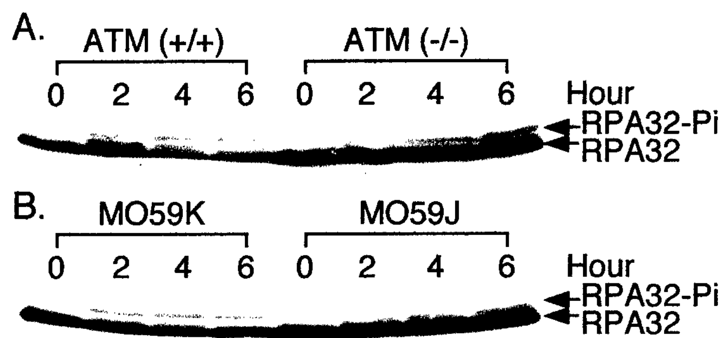

Figure 6

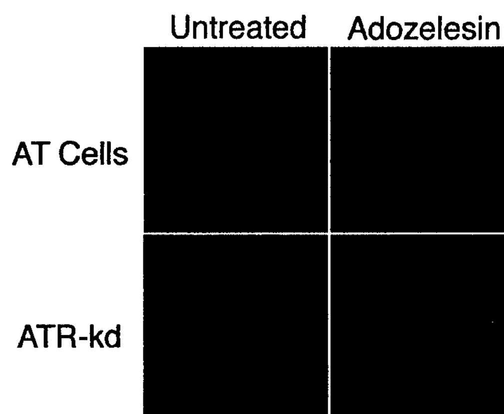

Figure 7

Figure legends:

Fig. 1. Formation of DNA damage-induced RPA and γ-H2AX foci in asynchronous cells. Exponentially growing HeLa cells were either mock-treated (A), or treated with 50 μM camptothecin (CPT) (B), 40 nM adozelesin (C) or 1 nM C-1027 (D) in the presence (right panels; 1 hr Aphidicolin) or absence (left panels; 0 hr Aphidicolin) of 2.5 μM aphidicolin. Cells were then fixed and stained with DAPI, RPA32-specific polyclonal antibody and monoclonal antibody against γ-H2AX.

Fig. 2. DNA damage-induced RPA foci in asynchronous cells labeled with BUdR. Asynchronous HeLa cells pulse labeled with BUdR were treated with the indicated agents. The total DNA is visualized by DAPI stain (left panels). Cells labeled with BUdR (S phase cells) are shown in the center panels. RPA stained with polyclonal antibody against RPA32 is shown in the right panels.

Fig. 3. DNA damage models for triggering RPA responses. Figure depicts three models for how different types of DNA lesions result in damage upon replication fork passage (A) DSBs can be easily recognized without replication fork passage. (B) Camptothecin-induced topoI:DNA adducts only generate DSBs and RPA and γ-H2AX DNA damage responses upon replication fork passage. (C) Similarly, poorly-recognized DNA damage in cells that progress into S phase results in stalled replication forks that only induce RPA and γ-H2AX DNA damage responses upon replication fork passage.

Fig. 4. Adozelesin-induced RPA and γ-H2AX foci. HeLa cells were treated with 40 nM adozelesin for the length of time indicated on the left of each panels. Cells were stained with RPA32-specific polyclonal antibody (red) and monoclonal antibody against γ-H2AX (green).

Fig. 5. Adozelesin-induced phosphorylation of Chk1, RPA and histone H2AX. HeLa cells were treated with 40 nM adozelesin for the length of time indicated on top of images. Migration of phospho- and underphospho-forms of Chk1 and RPA as well as phospho-H2AX are indicated at the right.

Fig. 6. Adozelesin-induced RPA phosphorylation in ATM or DNA-PK deficient cell lines. 40 nM adozelesin was used to treat 4 different cell lines: SV40-transformed human fibroblast cells ($ATM^{(+/+)}$); human fibroblast from AT patient ($ATM^{(-/-)}$); DNA-PK proficient cells (MO59K) and DNA-PK deficient cells (MO59J). Cells were lysed in SDS sample buffer and proteins were analyzed by SDS-PAGE and immunobloting.

Fig. 7. Adozelesin-induced RPA focus formation in ATM or ATR deficient cells. 40 nM adozelesin was used to treat $ATM^{(-/-)}$ cells or cells transfected with a dominant ATR kinase domain mutant. Polyclonal antibody against RPA32 was used to stain extraction-resistant RPA. The left panels (untreated) show regular S phase RPA staining.