Summary of Doctoral Dissertation (Doctoral Program (Science))

Department of Chemistry, Graduate School of Science
Author: Hosen Md Bayejid

論文題名：Characterization of Alovudine genotoxicity and its potential implementation as an anti-cancer drug

邦題：アロブジンの遺伝毒性特性と抗癌剤としての可能性の検討（英文）

Introduction

The chain-terminating nucleoside analogs (CTNAs) have been successfully used as an anti-viral and anti-cancer drug. The CTNAs are incorporated during replication and interfere with DNA replication of the virus and cancer cells, thereby restricting their proliferation. The CTNAs halt replication via two distinct mechanisms; The first mechanism is chain termination of the replication, in which CTNAs incorporated into nascent DNA inhibit subsequent polymerization reaction. In the second mechanism, the next round of DNA replication stalls on the CTNAs-incorporated template. The replication-arrest on the CTNAs-incorporated damaged template is released by translesion DNA synthesis (TLS) and homologous recombination (HR). The replication fork collapse due to the prolonged fork arrest on the damaged templates induces DNA double-strand breaks (DSBs) and such DSBs are repaired by HR and non-homologous end joining (NHEJ). In the HR-mediated DSB repair, BRCA1 protein plays a crucial role and mediates the end-resection of DSBs to initiate HR by counteracting the repressive function of 53BP1, an NHEJ factor.

Among CTNAs, the thymidine analog, alovudine (3'-deoxy-3'-fluorothymidine, FLT) is a potent inhibitor of reverse transcriptase and has been used as an anti-viral drug. The recent study by Yehudai et al showed that alovudine minimized the in vitro growth of acute myeloid leukemia (AML) cells by decreasing mt DNA synthesis. However, very little is known about the genotoxicity of alovudine and the molecular mechanisms underlying the interference of DNA replication by alovudine have not yet been elucidated. Here, we explored the DNA damage tolerance pathways responsible for the cellular resistance to alovudine using chicken DT40 cells. We showed the effects of alovudine incorporation into the genome DNA and mechanisms required for cellular tolerance to these alovudine-mediated effects.

Results and Discussion

Identification of DNA damage tolerance factors responsible for cellular tolerance to alovudine

To investigate the intrinsic mechanisms involved in the cellular tolerance to alovudine, we compared the cellular sensitivity to alovudine among parental wild-type cells and 23 chicken DT40 mutant cells deficient in different DNA repair pathways. We found that *BRCA1*$^{-/-}$, *BRCA2*$^{-/-}$, *FEN1*$^{-/-}$, *PARP1*$^{-/-}$ and *RAD18*$^{-/-}$ cells exhibited significantly higher sensitivity to alovudine than the wild-type cells, and among them, HR-deficient *BRCA1*$^{-/-}$ cells and BER-deficient *FEN1*$^{-/-}$ cells showed the highest sensitivity to alovudine. To specifically investigate the HR function of BRCA1, we disrupted the 53BP1 gene in *BRCA1*$^{-/-}$ cells and generated *BRCA1*$^{-/-}$/*53BP1*$^{-/-}$ double mutant cells, in which the HR function of BRCA1 is rescued. As expected, the loss of 53BP1 almost restored the hypersensitivity of *BRCA1*$^{-/-}$cells and *BRCA1*$^{-/-}$/*53BP1*$^{-/-}$ cells exhibited similar sensitivity to alovudine compared to wild-type and *53BP1*$^{-/-}$ cells. To identify the specific role of FEN1, we exposed other cells deficient in BER factors, among them only *FEN1*$^{-/-}$ cells showed hypersensitivity. Further, we have disrupted *53BP1*, *RAD18*, and *XRCC3* genes in *FEN1*$^{-/-}$ cells and found additive relationships of *FEN1* with *RAD18* and *XRCC3*. Interestingly disruption of the *53BP1* gene in *FEN1*$^{-/-}$ cells restored the hypersensitivity of alovudine to

wild-type level. These data suggest that FEN1 promotes cellular tolerance to alovudine through counteracting toxic roles of 53BP1 upon alovudine.

FEN1 antagonizes the interfering function of 53BP1 in removing mis-incorporated alovudine

To investigate the interfering effect of 53BP in removing mis-incorporated alovudine, we have integrated 53BP1 cDNA tagged with mCherry GFP protein in wild-type and *FEN1*$^{-/-}$ cells. After successful incorporation of mCherry tagged 53BP1 cDNA, we have treated these cells with 100 μM alovudine for 4 h and measured the number of 53BP1foci formation. The immunofluorescence data analysis disclosed that alovudine significantly augmented 53BP1 foci formation in *FEN1*$^{-/-}$ DT40 cells than in wild-type cells, suggesting that 53BP1 interferes removing mis-incorporated alovudine in *FEN1*$^{-/-}$ DT40 cells. We have also performed alkaline and neutral comet assays in wild-type, *FEN1*$^{-/-}$, *53BP1*$^{-/-}$ and *FEN1*$^{-/-}$/*53BP1*$^{-/-}$ cells to investigate whether the DNA damages are restored in *FEN1*$^{-/-}$ cells by the avolition of 53BP1 function in *FEN1*$^{-/-}$ cells. We revealed that alovudine augmented single-strand breaks (SSBs) and DSBs in *FEN1*$^{-/-}$ cells, which were restored in *FEN1*$^{-/-}$/*53BP1*$^{-/-}$ cells to wild-type and *53BP1*$^{-/-}$ levels. Collectively, these data indicate that FEN1 prevents 53BP1 to augment DNA damages during alovudine exposure. Further study might be needed to clarify the role of FEN1 and 53BP1 in the repair of DNA damage caused by alovudine.

BRCA1 and FEN1 avoids alovudine-induced chromosome aberrations and DSBs

Having established the role of BRCA1 and FEN1 in the cellular tolerance to alovudine, we next analyzed the effect of alovudine on the stability of chromosomes and the role of BRCA1 and FEN1. To this end, we employed chromosome analysis. We found that alovudine treatment significantly elevated the number of chromosome aberrations (CAs) in *BRCA1*$^{-/-}$ and *FEN1*$^{-/-}$ cells compared to wild-type cells, demonstrating the effects of alovudine in the stability of chromosomes and the pivotal role of BRCA1 and FEN1 in the avoidance of CAs upon alovudine exposure. Moreover, these data suggest that alovudine causes replication fork collapse and BRCA1 and FEN1 play roles in suppressing replication fork collapse.

To test this possibility, we measured the number of DNA lesions in *BRCA1*$^{-/-}$ and *FEN1*$^{-/-}$ cells at 12 h and 6 h after alovudine exposure, respectively. We defined cells that showed more than four γH2AX (a DNA damage maker) foci per nucleus as γH2AX foci positive cells, since the number of spontaneously arising γH2AX foci was less than four in most cells. The immunofluorescence data analysis revealed that alovudine significantly augmented DNA damage in *BRCA1*$^{-/-}$ DT40 cells than in wild-type cells, suggesting that replication fork collapse caused by alovudine is suppressed by BRCA1. On the other hand, the comet assay results revealed that tail DNA moments (an indicator of DNA damage) are significantly higher in *FEN1*$^{-/-}$ cells than the wild-type cells indicating larger number of DNA damage accumulation in *FEN1*$^{-/-}$ cells than in wild-type cells after alovudine exposure. Taken together, these data suggest that alovudine interferes with DNA replication during its incorporation and induces DNA damage, leading to CAs, and BRCA1 and FEN1 play influential roles in suppressing replication fork collapse.

Alovudine interferes with DNA replication after it is incorporated in genome DNA

To investigate the effect of alovudine on replication, we employed DNA fiber assay, which allows us to assess the effect of alovudine on replication fork progression. We measured the impacts of 50 μM of alovudine in *BRCA1*$^{-/-}$ and *FEN1*$^{-/-}$ cells and found that the replication speed was more pronouncedly slowed in both *BRCA1*$^{-/-}$ and *FEN1*$^{-/-}$ cells than in wild-type cells, indicating that BRCA1 and FEN1 remove incorporated alovudine from genome to avoid chain-termination effect. Additionally, we analyzed the *BRCA1*$^{-/-}$/*53BP1*$^{-/-}$ and *FEN1*$^{-/-}$/*53BP1*$^{-/-}$ cells to explore whether this effect is restored by the loss of 53BP1 and found that the defect in replication fork progression upon alovudine shown in

BRCA1$^{-/-}$ cells was not rescued while the defect shown in *FEN1*$^{-/-}$ cells was restored. On the other hand, the replication fork progression after alovudine was not affected in *XRCC2*$^{-/-}$ cells. These data suggest that BRCA1 and FEN1 have additional functions to mitigate the effect of alovudine in addition to their contributions in HR and BER respectively. Collectively, these data unveil the contribution of BRCA1 and FEN1 in the maintenance DNA replication, in which HR and BER functions are not required.

Pivotal contribution of BRCA1 in the maintenance of replication fork progression on the alovudine-incorporated template

To further investigate if HR function of BRCA1 as well as FEN1 is responsible for this function, we measured sister chromatid exchange (SCE) events as SCE is a hallmark of HR-mediated repair. We found the number of SCE was significantly reduced in *BRCA1*$^{-/-}$ cells compared to wild-type cells and *FEN1*$^{-/-}$ cells, suggesting the role of HR function of BRCA1 for the maintenance of replication on the alovudine-incorporated template. Moreover, we measured the numbers of SCE in wild-type, *53BP1*$^{-/-}$, *BRCA1*$^{-/-}$, *BRCA1*$^{-/-}$/*53BP1*$^{-/-}$ and *XRCC2*$^{-/-}$ cells. The loss of 53BP1 in *BRCA1*$^{-/-}$ cells restored the SCE almost to wild-type and *53BP1*$^{-/-}$ levels, while *BRCA1*$^{-/-}$ and *XRCC2*$^{-/-}$ cells showed significantly reduced SEC. Altogether, these findings revealed the critical contributions of HR-mediated bypass replication in the maintenance of replication on the alovudine-incorporated template.

Conclusion

In our present study, we have assessed the possible mechanisms underlying the genotoxic effects of alovudine. To our knowledge, our study was the first to quantitatively screen for alovudine sensitivity using several isogenic DNA repair-deficient cell lines. Interestingly, the evaluation of the sensitivity profile revealed that the *BRCA1*$^{-/-}$ and *FEN1*$^{-/-}$ cells showed more hypersensitivity to alovudine than all other DNA repair-deficient cell lines. We found that BRCA1 is pivotal for avoiding replication fork arrest after alovudine is incorporated into nascent DNA. A possible rationale is that BRCA1-CtIP and Mre11 might execute the excision of the incorporated alovudine from the DNA end to maintain continuous replication. We found that BRCA1 also contributes to the continuous replication on the alovudine-incorporated damaged template. We showed that the loss of BRCA1 reduced the kinetics of the replication fork progression using the alovudine-incorporated damaged template. We propose that HR function of the BRCA1 promotes template switch to bypass across the alovudine-incorporated template and thereby maintain replication. On the other hand, we revealed that FEN1 is also pivotal for avoiding replication fork arrest after alovudine is incorporated into nascent DNA while FEN1 plays very little role to maintain replication on the alovudine-incorporated damaged template. Further we showed that FEN1 antagonizes 53BP1 and prevent formation of SSBs and DSBs, thereby creates cellular tolerance against alovudine. In this study, we identified synergistic effects of alovudine exposure on *BRCA1*$^{-/-}$ and *FEN1*$^{-/-}$ genotypes. Mutations on the BRCA1 and FEN1 genes are frequently observed in different cancer including familial breast and ovarian cancers, and thus the implementation of new drugs such as alovudine that specifically and selectively kills BRCA1 and FEN1 deficient cells would add a new dimension to cancer therapy.