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Targeting base excision repair suggests a new therapeutic strategy of fludarabine for the treatment of chronic lymphocytic leukemia

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Fludarabine, a purine nucleoside analog, is widely used for the treatment of hematological malignancies including chronic lymphocytic leukemia (CLL).[1,2] Although fludarabine is the current standard treatment for CLL, the response to fludarabine is limited. Therefore, further understanding of the mechanism of fludarabine action is needed in order to develop new strategies to improve fludarabine therapeutic efficacy.

It is well known that the primary action of fludarabine is its incorporation into DNA. The incorporated F ara A serves as a poor substrate for DNA replication enzymes, leading to termination of DNA synthesis at the incorporated sites on the daughter strand. In addition, fludarabine induced inhibition of ribonucleotide reductase results in the depletion and imbalance of deoxynucleotide pools that are required for DNA repair and synthesis. This imbalance may subsequently favor the incor poration of F ara A itself, and other mismatched nucleotides such as uridine, into the newly synthesized DNA strand. These mismatched DNA base pairs would activate base excision repair (BER).

BER is the predominant DNA repair pathway that processes small base modifications generated by the exposure to either environmental mutagens or anticancer agents, and mis insertions from an imbalanced nucleotide pool. The BER pathway is proto typically initiated by a DNA glycosylase to remove a specific base lesion and generate an apurinic/apyrimidinic site (AP site). The resultant AP site is repaired by AP endonuclease, which incises the phosphodiester backbone of the DNA 5′ to the AP site, resulting in the formation of a 3′ hydroxyl residue and a 5′ deoxyribose phosphate group. In mammalian cells, completion of BER occurs via two pathways, either short patch (single nucleotide) or long patch (2 13 nucleotides) repair, depending on the ability to remove the 5′ deoxyribose phosphate and to complete repair synthesis.[3] BER is well known to be the most efficient repair mechanism to repair a variety of base lesions. However, although BER protects the cell from DNA damage, it also can render the cell resistant to anticancer agents that operate by creating DNA lesions that can be repaired by this pathway. To overcome BER conferred drug resistance, methoxyamine (MX) has been developed as an active inhibitor of BER. MX reacts specifically with the aldehyde group in the sugar moiety of the AP site, forming a MX bound AP site.[4] This modified AP site is resistant to the repair activity of AP endonuclease, resulting in the persistence of the DNA lesions. MX has been demonstrated to enhance the therapeutic efficacy of different alkylating therapeutic agents[5,6] and is currently being evaluated in phase I clinical trials.

The present work is aimed to determine whether and how incorporated fludarabine is repaired by BER pathway and whether MX is capable of potentiating the cytotoxicity of fludarabine via its ability to block BER pathway. We are particularly interesting in determining the impact of BER activity on antitumor effect of fludarabine in human primary CLL cells.

Using oligonucleotides containing F ara A:Thymidine (F:T) mismatched base pairs (Figure 1a), we examined the ability of several DNA glycosylases to excise incorporated F ara A. We found, via *in vitro* and *ex vivo* assays, that uracil DNA glycosylase (UDG, which is encoded by *UNG* gene) was capable of excising incorporated F ara A to generate a cleaved

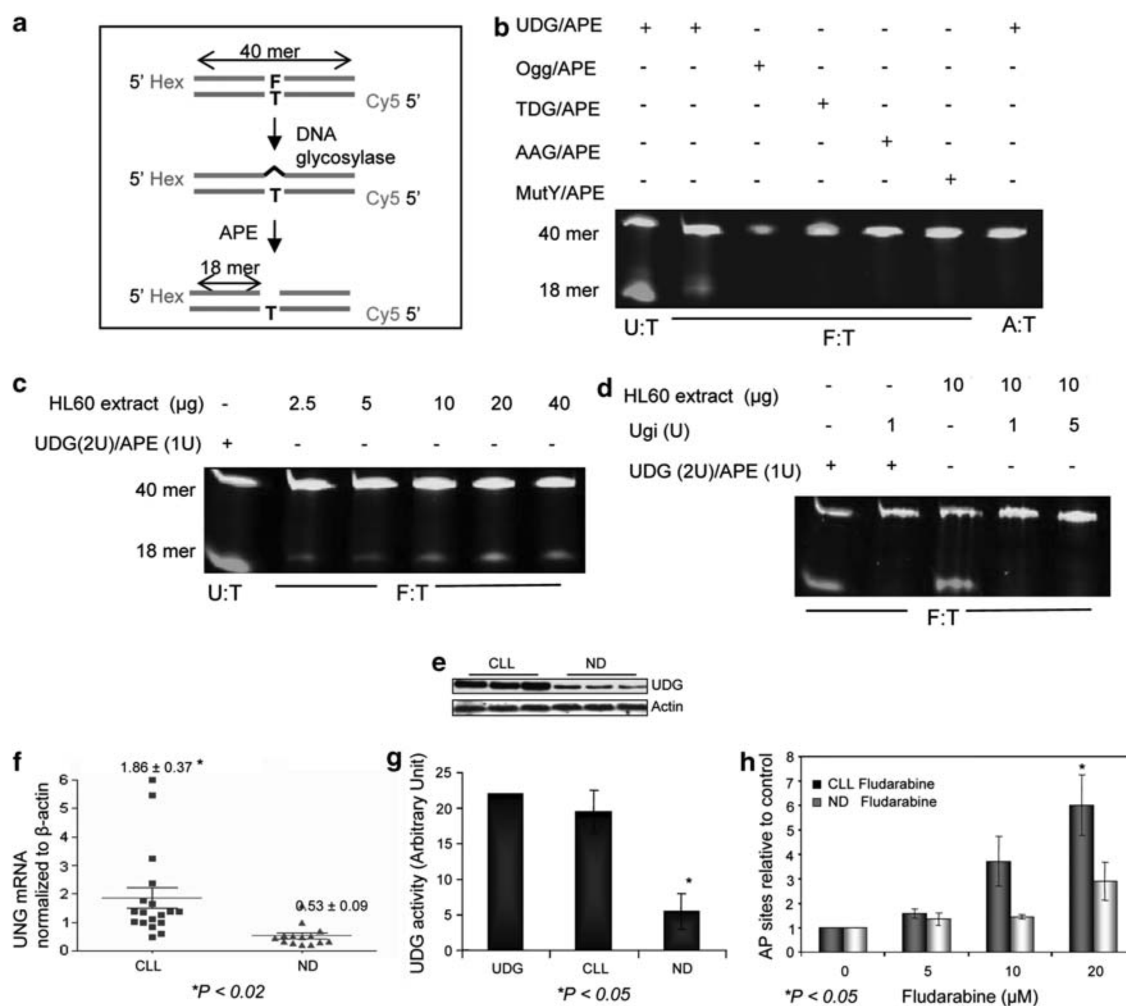

Figure 1 UDG removes incorporated fludarabine from DNA. (**a**) Schematic diagram represents the preparation of oligonucleotide substrates containing F ara A:Thymidine (F:T) mispairs and the cleavage reaction by DNA glycosylase/AP endonuclease (APE) when reacted with the oligonucleotide substrates. (**b**) Oligonucleotide duplexes containing F:T were incubated with 5 DNA glycosylases (UDG, OGG (8-oxoguanine-DNA glycosylase), TDG (thymine DNA glycosylase), AAG (Alkyl adenine glycosylase) and MutY (the *Escherichia coli* adenine glycosylase)) at 37 °C for 1 h, followed by incubation with APE at 37 °C for 30 min. Reaction products were resolved by electrophoresis through denaturing 20% polyacrylamide gels. UDG has activity to excise F Ade, but has no activity to cleave the normal substrates containing A:T. (**c**) Oligonucleotide duplex containing F:T base pair was incubated with HL60 cell extracts (2.5 40 μg) at 37 °C for 30 min. (**d**) UDG activity in HL60 cell extract was inhibited by uracil glycosylase inhibitor (Ugi was pre incubated with cell extracts at 37 °C for 30 min). (**e**) CLL and normal bone marrow cell lysates were subjected to western blot analysis with UDG specific antibodies. (**f**) Levels of UDG mRNA in CLL (*n* 18) versus normal lymphocytes and bone marrow (normal donor (ND), *n* 14) samples were determined by quantitative reverse transcription PCR (*$P<0.02$). (**g**) Comparison of UDG enzymatic activities of CLL and normal lymphocytes (*$P<0.05$). UDG activity was determined based on the cleaved products obtained after oligonucleotide substrates were incubated with 5 μg cell extracts and quantified by using ImageQuant software (GE HealthCare, Piscataway, NJ, USA). (**h**) The dose dependent relationship between the numbers of AP sites and the concentrations of fludarabine. CLL cells and normal lymphocytes were cultured in complete RPMI 1640 containing 10% fetal calf serum, 2 mM L glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). CLL and normal lymphocytes were treated with fludarabine alone (0 20 μM) for 24 h. Genomic DNA was extracted and AP sites were measured using aldehyde reactive probe reagent (*$P<0.05$). Results are representative of at least three experiments. Ugi, uracil glycosylase inhibitor.

fragment (Figure 1b). As shown in Figure 1c, the production of 18 mer fragments, produced through UDG mediated excision of F ara A, was proportionally increased with increasing concentrations of HL60 cell extracts, demonstrating a direct relationship between UDG activity and the levels of excised F ara A. However, the UDG cleavage activity was abolished when the cell extracts were pre incubated with uracil glycosy lase inhibitor (Ugi), a bacteriophage encoded short polypeptide that depletes UDG activity through the binding and formation of a tight UDG:uracil glycosylase inhibitor complex (Figure 1d). The results confirm that the cellular activity of UDG has an important role in the removal of incorporated fludarabine from DNA.

We next examined both UDG expression and activity in primary CLL cells obtained from 18 patients. Interestingly, we found that UDG was highly expressed in CLL cells. The levels of both UDG protein (Figure 1e) and mRNA (Figure 1f) in CLL cells were higher (6.7 and 4.3 fold, respectively) than that in normal cells (lymphocytes and bone marrow cells). As expected, the higher activity of UDG in CLL cells (Figure 1g) produced more AP sites, which was proportionally increased with the concen trations of fludarabine. In contrast, only a moderate increase in

AP site formation was detected in normal cells when treated with high concentrations of fludarabine (Figure 1h). Owing to the fact that AP site formation is the results of fludarabine removed from DNA by UDG, the AP sites detected in CLL cells indicate that fludarabine can actively incorporate into DNA. Most importantly, these results support the findings that subclonal cell populations of CLL are substantially replicating.[7] In these cells, cellular BER mechanism presumably controls the cytotoxic effect of fludarabine.

The identification of the role of UDG in the removal of F ara A from DNA not only uncovers an important mechanism responsible for the cellular resistance to fludarabine, but also provides a rationale for fludarabine MX combinatorial therapies. MX has been shown to potentiate the cytotoxicity of several alkylating therapeutic agents[3,4] through the covalent binding of an AP site. This biochemical reaction converts a repairable AP site into a structurally modified AP site, which is refractory to the lyase activity of AP endonuclease and polymerase β, and consequently blocks the downstream effectors of the BER pathway. The MX bound AP sites subsequently stall DNA replication, induce severe metaphase chromosomal aberrations (that is, chromosome fragmentation and sister chromatin exchange events), and trigger apoptotic death.[5,6]

Consistent with previous results, the combination of fludarabine and MX significantly induced DNA double strand breaks quantified by the comet assay (Figures 2a and b). This result was

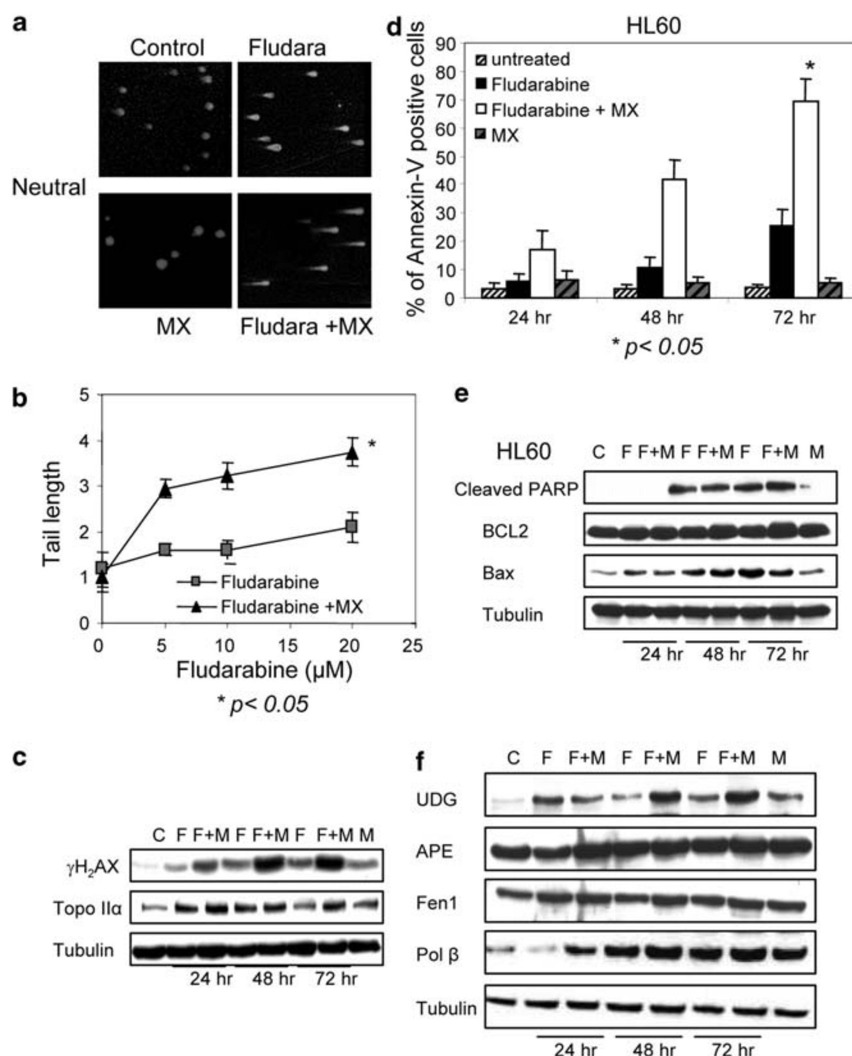

Figure 2 MX enhances cytotoxicity of fludarabine in leukemia cells. (**a**) Comet images assayed by neutral electrophoresis show that increased DNA double stranded breaks detected in cells treated with combination of fludarabine and MX. HL60 cells were treated with fludarabine alone (5 μM) or fludarabine plus MX (3 mM) for 4 h. (**b**) Tail length of the comet detected by neutral electrophoresis in HL60 cells after treatments with fludarabine alone (0 20 μM) and fludarabine plus MX (3 mM, $^*P<0.05$, compared with the treatment with fludarabine alone). (**c**) HL60 cells were continuously treated with drugs (5 μM fludarabine alone and fludarabine plus 3 mM MX) and collected at 24, 48 and 72 h. Induction of expression levels of γH2AX and topoisomerase IIα proteins was detected by western blotting. (**d**) The percentage of Annexin V positive cells induced by fludarabine alone and fludarabine plus MX at 24, 48 and 72 h ($^*P<0.02$ compared with the treatment with fludarabine alone). (**e**) Induction of the cleaved PARP and Bax proteins was detected by western blotting in cells after treatments with fludarabine alone and in combination with MX. No changes in Bcl2 protein levels. (**f**). Induction of BER proteins was analyzed by western blotting in cells after drug treatment over 72 h. Samples analyzed in experiments (**c** **f**) were cells collected from the same treatments: control (lane 1), fludarabine alone (24, 48 and 72 h, lanes 2, 4, 6), fludarabine plus MX (24, 48 and 72 h, lanes 3,5,7), MX alone (72 h exposure, lane 8). Results are representative of at least three experiments. APE, AP endonuclease; Pol β, polymerase β.

supported by the finding that γH2AX, a well know marker of DNA double strand break, was highly induced in cells treated with the combination of fludarabine and MX, when compared with treatment with fludarabine alone (Figure 2c). In agreement with previous work,[4] increased levels of γH2AX occurred concomitantly with the upregulation of topoisomerase II α (topo II α; Figure 2c), suggesting that MX bound AP sites act as a topo II α poison, inducing topo II α mediated DNA double strand break.[6] Apoptosis analysis performed in HL60 cells revealed that treatment with fludarabine alone resulted in low levels of apoptotic cell death. A possible explanation for this is that the p53 deficiency in HL60 cells results in decreased p53 dependent apoptosis (Figure 2d). In contrast, cotreatment of fludarabine with MX significantly increased the percentage of apoptotic cells as evidenced by the level of Annexin V positive labeled cells. At 72 h, ~70% of cells were more likely to undergo apoptosis (*$P<0.02$). The measurements of proteolytic cleavage of PARP by caspases, a hallmark of apoptotic cell death, demonstrated a time dependent apoptotic death, which was associated with the induction of the proapoptotic protein Bax (Figure 2e) Altogether, the data indicate that MX enhanced DNA lesions (MX bound AP sites and double strand breaks) contribute to the propagation of p53 independent apoptotic death. As p53 is the most commonly mutated tumor suppressor in human cancer, this finding highlights the importance of combined therapy with MX. Indeed, various investigators have shown that B cell Chronic Lymphocytic Leukemia and Acute Myeloid Leukemia patients with mutations in p53 display fludarabine resistance.

The involvement of BER in response to the DNA damage induced by the treatment with fludarabine alone or in combination with MX was evident from the induction of several BER proteins, including UDG, polymerase β and FEN1 (Figure 2f). UDG protein was significantly upregulated after drug treatments, particularly in cells treated with the combination treatment of fludarabine and MX. On the basis of previous observation that UDG has a higher affinity for the products at the AP site than the actual substrate,[8] it has been proposed that subsequent to a base release, UDG remains bound to the AP site to protect the cell until the AP site is transferred to the downstream BER pathway enzymes, AP endonuclease and polymerase β. Although the upregulation of UDG in response to fludarabine and MX occurs at the transcriptional level (data not shown), it is also possible that the persistence of MX bound AP sites results in the accumulation of UDG at un repairable MX bound AP sites. In agreement with previous report,[3] the elevated polymerase β, the limiting factor for short patch BER, and FEN1, the protein responsible for long patch BER process, suggest that MX AP sites impact both short and long patch BER. Although the mechanisms for the regulation of BER proteins by fludarabine treatment are not yet clear, these results suggest that BER proteins have an adaptive response to DNA damage that is exaggerated by the combination of fludarabine and MX.

MX was capable of potentiating the antitumor effect of fludarabine, *in vitro* and *in vivo*. In agreement with the induction of apoptosis, a remarkable inhibition of cell growth was observed by the combined treatment of fludarabine and MX

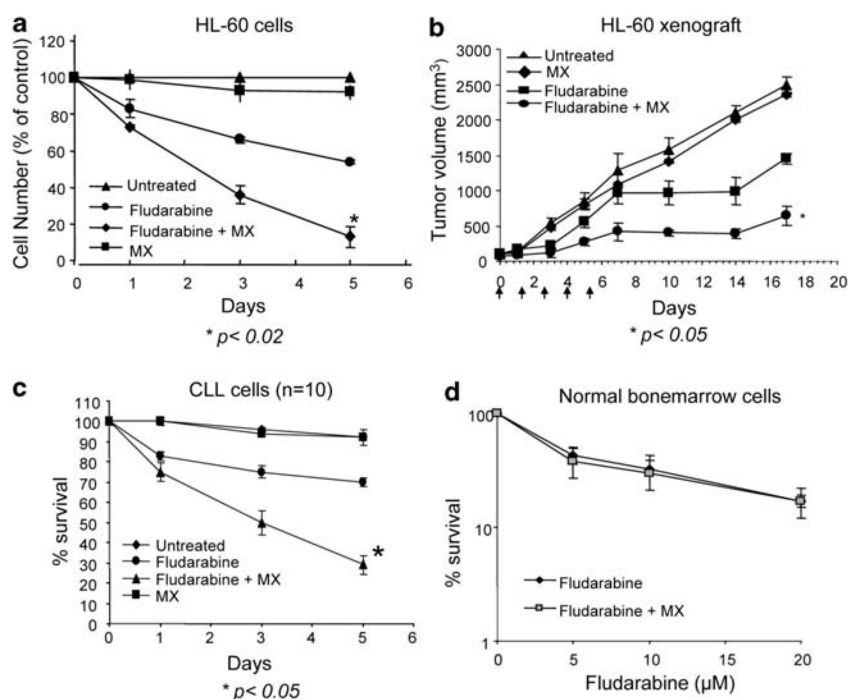

Figure 3 MX augments the chemotherapeutic effect of fludarabine *in vitro* and *in vivo*. (**a**) Growth inhibition induced by fludarabine and the combination with MX in HL60 cells. HL60 cells were seeded in complete growth medium in a 6 well plate (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) at a density of 5×10^5 cells per well. Cells were treated with fludarabine (5 μM) or fludarabine plus MX (3 mM). Cell viability was determined by trypan blue exclusion every 24 h for 5 days. (**b**) MX enhanced antitumor effect of fludarabine in xenografts (HL60). (The arrowhead indicates drug treatment daily, *$P<0.05$ compared with the treatment with fludarabine alone). (**c**) Cytotoxicity of fludarabine or fludarabine plus MX in primary CLL cells detected by trypan blue exclusion. CLL cells were treated with 5 μM fludarabine and fludarabine plus 3 mM MX. Cell viability was expressed as percentage of untreated control. The results are representative of 10 primary CLL samples. (**d**) Comparison of the cytotoxicities induced by fludarabine alone and the combination of fludarabine and methoxyamine in normal bone marrow cells assayed by clonogenic formation.

in both HL60 and CLL cells (Figures 3a and c). MX enhanced fludarabine cytotoxicity by threefold (*$P<0.05$) when compared with fludarabine treatment alone. In addition, MX potentiated the therapeutic efficacy of fludarabine as evidenced in HL60 xenografts in nude mice. As shown in Figure 3b and a, moderate sensitivity to fludarabine alone was observed in HL60 xeno grafts; however, significant inhibition of tumor growth was seen after the combined fludarabine plus MX treatment. At 17 days, HL60 xenografts treated with PBS (control group) had a mean tumor volume of $2500 \pm 106\,mm^3$ compared with a mean tumor volume of $1456 \pm 76\,mm^3$ and $643 \pm 142\,mm^3$ (*$P<0.05$) in mice treated with either fludarabine alone or with the combined fludarabine plus MX treatment, respectively. These results demonstrate that the disruption of the BER by MX increased the magnitude of the response to fludarabine. MX alone did not affect tumor growth, displaying a similar growth rate to that observed in control group. At these doses, mice did not present any evidence of toxicity as evaluated by measurements of body weight and whole blood toxicity analysis. To further confirm that MX enhanced cytotoxicity of fludarabine is associated with the cellular levels of UDG and topo II α, normal bone marrow cells were treated with fludarabine alone and in combination with MX. Results showed that MX did not appear to increase the cytotoxicity observed with fludarabine alone in normal bone marrow cells, presumably, because these cells express lower levels of both UDG and topo II α compared with tumor cells.[7] These results strongly suggest that the induced killing effect of the combination treatment of fludarabine and MX would be selective toward tumor cells, which would be relatively protective of normal bone marrow cells.

In summary, we demonstrate that (i) a new mechanism, the BER pathway, is involved in processing incorporated fludar abine, as well as mis incorporated uridine in DNA through the enzymatic activity of UDG; (ii) MX binding of AP sites generated directly and indirectly by fludarabine induces un repairable DNA damage that can block the BER pathway; and (iii) MX potentiates the therapeutic efficacy of fludarabine, allowing for the possibility of a novel therapeutic strategy to combine inhibitors of BER with fludarabine for clinical treatment. Targeting BER as a target based therapeutic strategy can be extended to the combination of MX with a number of other drugs that incorporate into DNA either by acting as nucleotide analogs or through the manipulation of the nucleotide pools.

Conflict of interest

SL Gerson & L Liu have conflict of interest. Tracon Pharmaceuticals Inc licenses intellectual property of Methoxyamine (MX).

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