The impact of expression of BER proteins on therapeutic effect of combining temozolomide with methoxyamine in melanoma cells

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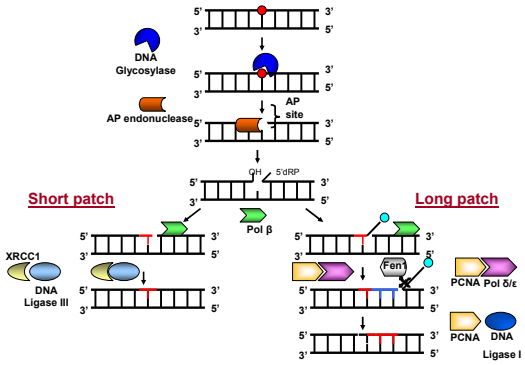
Abstract

Base excision repair (BER) is a highly coordinated cellular biochemical system essential to cell survival. Variability of expression of BER proteins may affect the dynamics of the repair pathway or change the repair rate that determines the cellular sensitivity to the cytotoxicity exerted by alkylating agents. In the present study, we examined the relationship between the expression of BER proteins and therapeutic effect of temozolomide (TMZ) and the combination of TMZ and methoxyamine (MX), a BER inhibitor, in melanoma cells in vitro and in xenografts. The levels of BER proteins (including MPG, UDG, APE, and Pol β) were measured by western blotting assay in 4 cell lines, SkmCl-1, WM164, A375, and WM9. Results revealed that BER proteins were coordinately and differentially expressed in these melanoma cell lines, higher in A375 and much lower in WM164 cells. There were no significant differences in MGMT and MLH1-MSH2, two well known TMZ resistant factors in these two cell lines. To determine the role of BER proteins in TMZ cytotoxicity, clonogenic survival studies were performed in A375 and WM164 cells. The sensitivity to TMZ alone in these two cell lines was assessed by IC90 value produced by survival assay, showing 490 μM in A375 and 570 μM in WM164 cells. When cells were treated with the combination of TMZ and MX, MX efficiently potentiated TMZ cytotoxicity by 3 folds in A375 but failed to enhance the killing effect of TMZ in WM164 cells. Similar results were observed in xenografts. In xenograft study, MX enhanced TMZ antitumor effect, resulting in tumor growth delays of 13 days in mice receiving the combination of TMZ (80 mg/kg) and MX (2 mg/kg) i.p daily for five days in comparison to 6 days in mice treated with TMZ alone. No enhancement of TMZ-antitumor activity by MX was observed in mice carrying WM164 melanoma xenografts. The results suggest that the failure of TMZ-potentiation by MX in WM164 cells was related to the low activity of BER proteins, particularly low levels of MPG DNA glycosylase. Deficiency of the BER proteins would decrease the formation of AP sites, the targets for MX action. These results indicate that BER proteins may be differentially expressed in tumor cells, which particularly impact the therapeutic effect of combining TMZ with MX

Background and Hypothesis

Base excision repair pathway

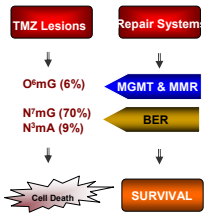


Hypothesis:

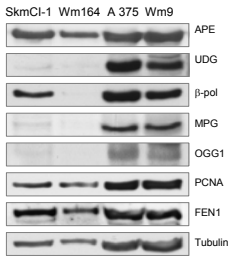
Variability of expression of BER proteins may affect the dynamic of repair pathway that will determine the cellular sensitivity ti the cytotoxicity exerted by alkylating agents

Results

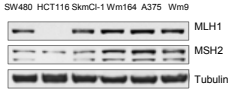
Differential expression of key components of repair systems in melanoma cell lines



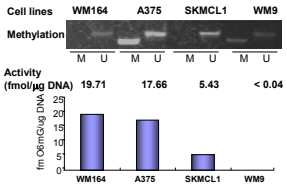
I. BER proteins expression



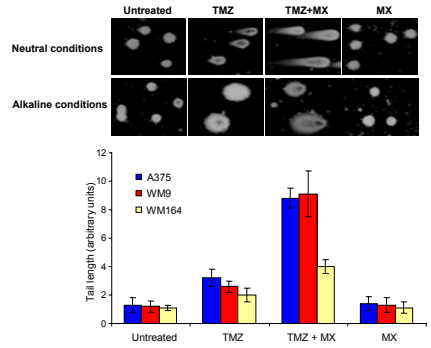
II. MMR expression



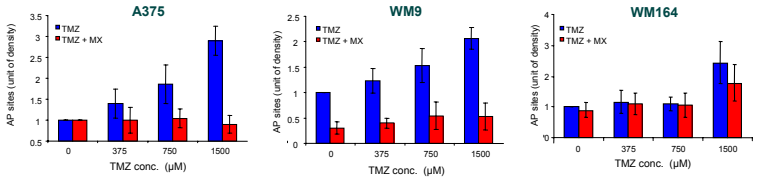
III. MGMT expression and epigenetics



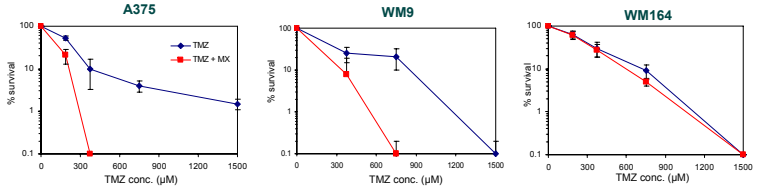
DNA strand brakes formation induced by TMZ plus MX treatments correlates with expression levels of BER protein

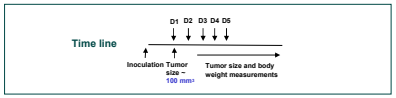


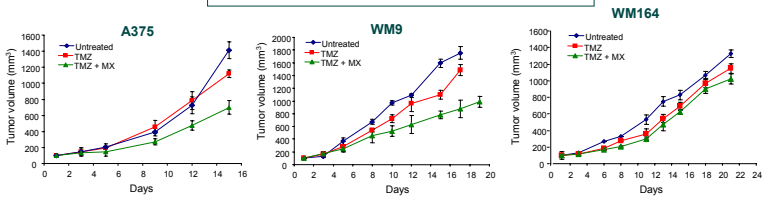
BER activation and subsequent AP sites formation is dependent of expression of key components

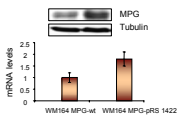


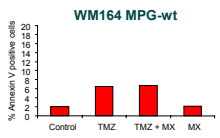
MX potentiation of TMZ cytotoxicity in cell lines and xenografts is dependent on BER expression

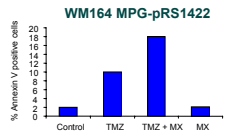












Summary:

- MX failure to potentiate TMZ cytotoxicity in WM164 correlates to low BER protein expression
- Higher BER expression (WM9 and A375) leads to formation of AP sites after TMZ treatments, resulting in formation of DNA strand breaks an cell death
- Increased expression of MPG (by genetic manipulations) resulted in enhancement by MX of TMZ cytotoxicity

Enhancement of decitabine cytotoxicity by methoxyamine via inhibition of base excision repair

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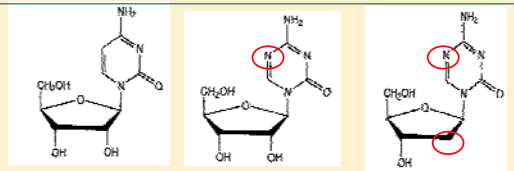
Abstract

Decitabine (5aza-2'deoxycytidine) is a nucleoside analog used for the treatment of hematological malignancies. Previously, it was shown that the cytotoxic effect of low dose decitabine treatment is due to incorporation of its active metabolite, 5aza-2'deoxycytidine triphosphate, into DNA leading to inhibition of DNA methylation by binding irreversibly to DNA methyltransferases. We hypothesized that incorporated 5aza-2'deoxycytidine (or its deaminated analog, 5aza-2'deoxuridine) into DNA is also recognized and processed by the base excision repair (BER) pathway. In this case, inhibition of BER by methoxyamine (MX) would potentiate the cytotoxicity of decitabine. We evaluated role of BER in decitabine cytotoxicity in colon cancer, melanoma cells, and primary acute myelogenos leukemia (AML) cells. Decitabine-induced abasic sites (AP-sites) were increased proportionally with dose and duration of exposure. MX reduced the number of available AP-sites up to 80% indicating formation of stable MX-bound AP-sites that have the potential to interrupt BER pathway. A similar correlation between decitabine dose and the AP-sites formed was observed after *in vitro* exposure of primary AML cells to increase concentrations of decitabine. MX was able to bind a significant percentage (up to 60%) of these AP sites. Decitabine cytotoxicity was potentiated by MX. Cell survival assays demonstrate a 4-fold decrease in the IC50 for decitabine when cells were co-treated with MX (IC50 dec = 4 μM and IC50 Dec+ MX = 1 μM). Apoptotic cell death measurements using Annexin V staining showed a 5-fold increase in cell death following decitabine and MX treatments. These events were accompanied by a concomitant increase in cleavage of PARP, and in γH2AX. Moreover, MX enhanced decitabine-induced antitumor effect in mice bearing A375 human melanoma xenografts, as measured by tumor growth delay: 7 days (0.5 mg/kg decitabine) versus 14 days (0.5 mg/kg decitabine plus 2 mg/kg MX).These studies not only suggests for the first time the role of BER in the processing of incorporated decitabine, but they also provide insights into a new and promising cancer therapeutic strategy of combining decitabine with MX to block BER.

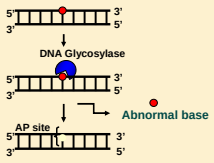
Background and Hypotheses



Cytidine | **5-AzaCytidine** (DNA, RNA) | **5-Aza DeoxyCytidine (Decitabine)** (DNA)

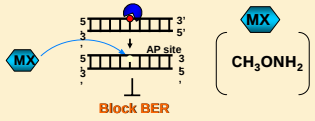
Hypothesis 1:

Incorporated 5-Aza-deoxycytidine is a substrate for base excision repair pathway which process it as an abnormal base.

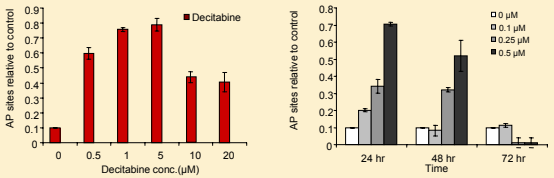


Hypothesis 2:

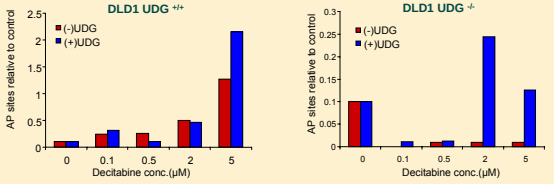
Methoxyamine blocks BER response and enhances cytotoxicity of Decitabine.



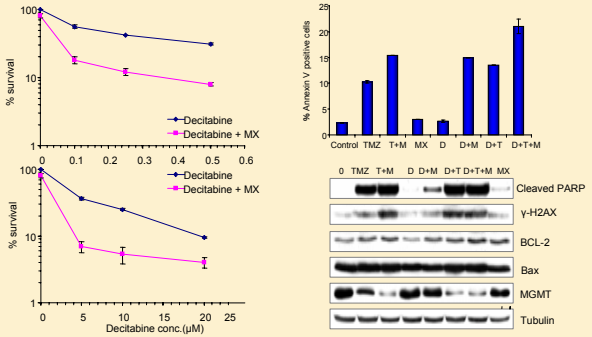
Dose- and time dependent abasic sites are formed following Decitabine treatments



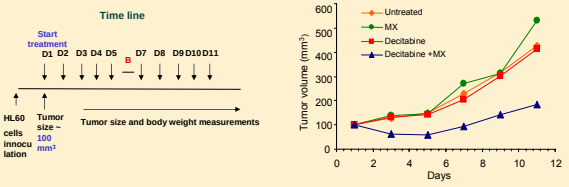
UDG may play a role in BER response to Decitabine treatments



MX enhances Decitabine-induced apoptotic cell death



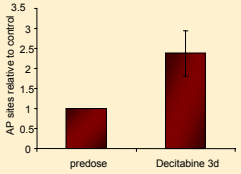
MX augments therapeutic effect of Decitabine in human xenografts



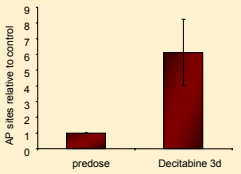
Results

Measurement of AP sites in peripheral primary lymphocytes pre and post- treatments with Decitabine

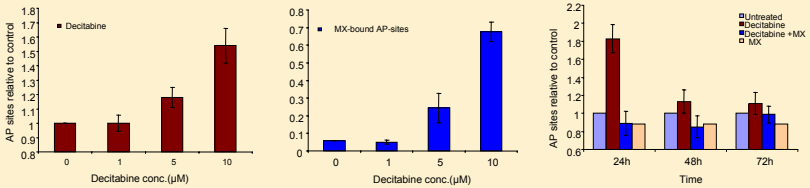
1. Clinical settings: AML; Decitabine = 40 mg IV over 1 hr, daily



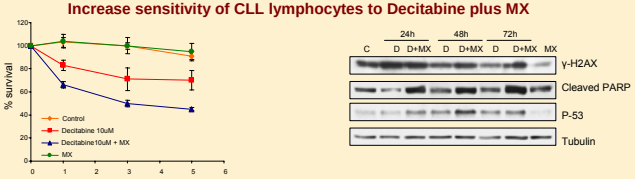
2. Clinical settings: CLL; Decitabine = 40 mg IV over 1 hr, daily



MX binds the AP sites formed by in vitro exposure of CLL lymphocytes to Decitabine

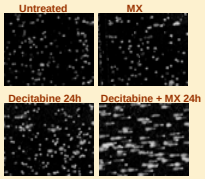


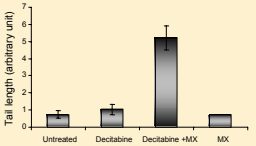
Increase sensitivity of CLL lymphocytes to Decitabine plus MX



MX enhances DNA strand breaks induced by Decitabine (mechanism for cell death)

Comet assay: Alkaline conditions





Summary:

- MX binds AP sites formed by Decitabine
- MX enhances DNA strand breaks formed by Decitabine
- MX augments antitumor effect of fludarabine in cancer cell lines, primary cells and human xenografts

Induction of uracil DNA glycosylase (UDG) in human cancer cells in response to antimetabolites and methoxyamine

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ABSTRACT

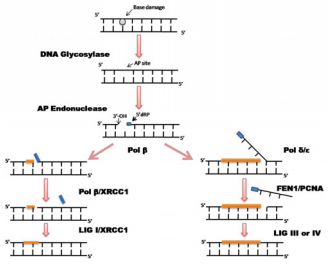
Background: Antimetabolites are used in cancer therapy to disrupt RNA and DNA production and induce cell death. Many DNA lesions created by antimetabolite chemotherapy are repaired by base excision repair (BER). We have previously reported that methoxyamine (MX), which inhibits BER by binding and stabilizing abasic (AP) sites after glycosylase removal of abnormal bases, enhances the cytotoxicity of the antimetabolite fludarabine, a purine analog, in human leukemia (Jurkat) cells. This MX-potentiated cytotoxicity was also observed in lung, breast and colon cancer cells after treatment with pemetrexed, an antifolate antimetabolite. Cytotoxicity of pemetrexed has been attributed to thymidylate synthetase (TS) inhibition which causes reduction of dTTP levels with a concomitant rise in dUTP. In this milieu, uracil is aberrantly incorporated into the genome, removed by uracil DNA glycosylase (UDG), and reincorporated during DNA replication. To better understand antimetabolite-induced tumor cell killing, we sought to determine the role of base excision repair (BER) and the induction of the enzyme uracil DNA glycosylase following fludarabine and pemetrexed treatment.

Materials & Methods: We examined the effects of antimetabolite therapy alone and in combination with MX on the induction of the BER protein, UDG, in established human cancer cell lines. Human leukemia cells (Jurkat) were treated with fludarabine (5uM) alone or in combination with MX treatment (3mM). Human lung cancer cells (H460 and A549) were treated with pemetrexed (100-400uM) alone and in combination with MX (6mM) for 24 hr. UDG protein and mRNA expression was m onitored by western blotting and RT-PCR analysis, respectively. Normal fluctuation of UDG with cell cycle stage was established in H460 cells by monitoring protein and mRNA levels at time points between 0-48 hours post serum starvation release. Cell cycle progression was determined by propidium iodide mediated FACS analysis.

Results: Immunoblot data indicated modest enhancement of UDG protein level following fludarabine treatment (~1.5 fold) and more significant enhancement with MX adjunct therapy (2-3 fold). RT-PCR similarly revealed induction of UDG transcript level (2-3 fold) in response to fludarabine and MX treatment. Significant induction of UDG protein was observed in cells treated with pemetrexed and MX, resulting in a 2-fold increase in UDG enzymatic activity measured by UDG cleavage assay. Meanwhile, a concomitant increase in level of γH2AX and cleaved PARP was seen in the cells. Therefore, UDG induction functioned as a mechanism of cell protection and resulted in enhanced DNA repair. However, increased UDG activity also enhanced AP site production. Normally, UDG and BER process these lesions but in the presence of MX, BER is blocked, leading to cell death. In this way, pemetrexed -induced nucleotide pool imbalance, results in futile cycles of uracil incorporation into DNA and uracil removal.

BACKGROUND

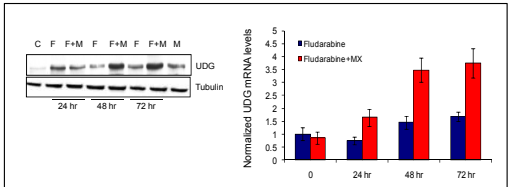
Inducible DNA repair is required by cells to counteract the harmful effects of continuous exposure to environmental and endogenous DNA damaging agents. Base excision repair (BER) is responsible for handling a diverse array of DNA lesions arising as a result of intrinsic DNA instability or reactive species of both endogenous and exogenous origin. The BER pathway is comprised of three major steps: damage recognition and excision by a damage specific DNA glycosylase; phosphodiester bond cleavage and generation of a single strand break by AP endonuclease(APE); nucleotide addition by DNA polymerase; and gap ligation by DNA ligases. In BER, the repair of all lesions is funneled through the glycosylase-mediated generation of apurinic/apyrimidic (AP) sites which are resolved as a result of downstream pathway activity. Published accounts suggest induction of various DNA glycosylases as one component of the cellular response to DNA damage. Though not well understood, this induction may occur through changes in protein localiazation,[1] transcriptional regulation and new protein synthesis.[2,3] Glycosylase induction may enhance lesion repair through increased excision of modified bases. However, increased glycosylase expression may also result in AP site accumulation which can lead to double strand breaks (DSBs)[4] or random base incorporation during semi conservative replication. Uracil DNA glycosylase (UDG) is the major DNA glycosylase for the removal of uracil, arising from incorporation of uracil during replication and spontaneous deamination of cytosine throughout the genome.[5,6] Here, we have identified induction of UDG in human cancer cells in response to the antimetabolites fludarabine and pemetrexed and we have initiated studies to determine both the mechanism and consequence of UDG induction in cancer cells following treatment with these antimetabolites.



RESULTS SUMMARY

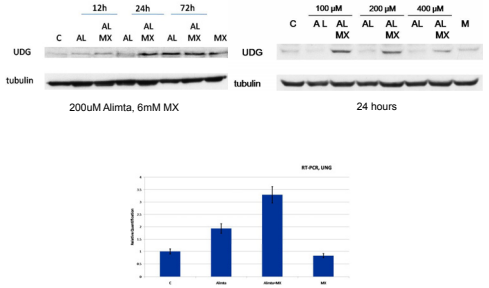
- Protein and mRNA induction of UDG is observed in human leukemia cells following fludarabine treatment and is enhanced with methoxyamine co-treatment.
- Induction of UDG is observed at the protein and mRNA level following pemetrexed treatment in human lung cancer cells (H460) and is enhanced with methoxyamine co-treatment.
- Modest fluctuations in UDG expression are observed with normal cell cycle stage progression
- Pemetrexed treatment results in early S phase arrest and AP site formation which is positively correlated with UDG induction.
- Pemetrexed treatment causes concomitant increased in γ-H2AX and cleaved PARP indicating strand break formation and induction of apoptosis, respectively.

Fludarabine causes UDG induction that is potentiated by methoxyamine



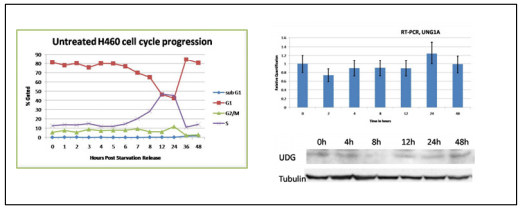
Induction of UDG in leukemia (Jurkat) cells treated with 5uM Fludarabine and 6mM MX combination for 24-72 hours

Pemetrexed causes induction of UDG that is potentiated by methoxyamine

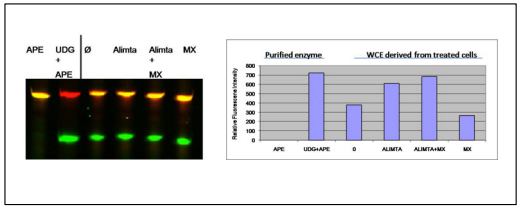


Dose and treatment time dependent induction of UDG in human lung cancer cells (H460) treated with 200-400uM pemetrexed and 6mM methoxyamine for 12-72 hours.

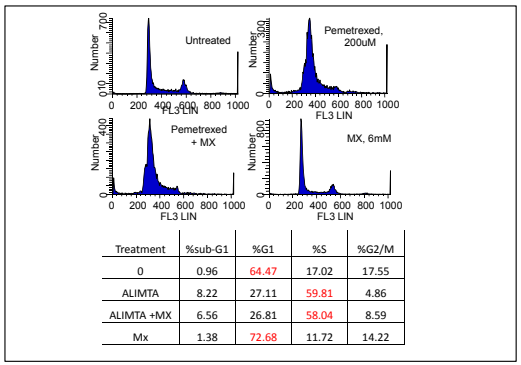
Moderate fluctuation of UDG expression with cell cycle stage



UDG expression correlates with *in vitro* enzyme cutting activity

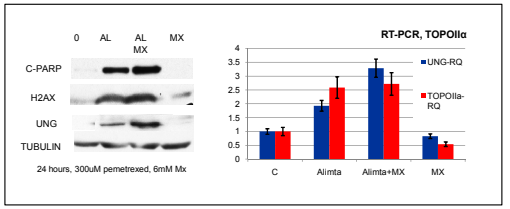


Pemetrexed treatment results in early S phase arrest that is positively correlated with UDG induction



Treatment	%sub-G1	%G1	%S	%G2/M
0	0.96	64.47	17.02	17.55
ALIMTA	8.22	27.11	59.81	4.86
ALIMTA +MX	6.56	26.81	58.04	8.59
Mx	1.38	72.68	11.72	14.22

Pemetrexed Induction of UDG corresponds to concomitant increases in expression of γ-H2AX, cleaved PARP and Topoisomerase II α (TOPOII α)



CONCLUSIONS

We have illustrated that the expression of human Uracil DNA glycosylase (UDG) mRNA and protein is enhanced following treatment with the antimetabolites fludarabine and pemetrexed. Cell cycle analysis of untreated H460 cells indicated a peak in S-phase percentage at 12 and 24 hours post synchronization. However, analysis of UDG expression in these cells does not suggest significant enhancement of UDG mRNA or protein as a consequence of normal cell cycle stage progression. Contrarily, cells treated with pemetrexed displayed early S-phase arrest and significant enhancement of UDG protein and transcript. Induction of UDG is accompanied by enhancement of strand break formation and caspase activation as measured by γ-H2AX and cleaved PARP detection, respectively. Furthermore, a concomitant increase in topoisomerase II alpha (topoIIα) mRNA implicates topoIIα mediated cleavage in strand break formation. Therefore, despite modest increases in UDG cutting (a measurement of BER capacity), induction of UDG is insufficient to reverse cytotoxicity and prevent cell death. Indeed, UDG induction appears to be coupled with evidence of DNA strand breaks and apoptotic signaling. Methoxyamine blockade of BER further exacerbated this DNA damage response and potentiated cytotoxicity of both fludarabine and pemetrexed and also potentiated UDG expression. Together, these data suggest that UDG induction may occur initially as a compensatory mechanism of cell protection from DNA damage. However, UDG induction also appears to contribute to increased AP site formation and MX-bound AP sites. These lesions act as topoIIα poisons, leading to topoIIα-mediated doble strand breaks (DSB), detected by γ-H2AX, a well known marker of DSB. In this way, UDG induction may contribute to the cytotoxicity of fludarabine and pemetrexed in human cancer cells. Ongoing work involves identifying factors which regulate the transcription of UNG following DNA damage and determining the effects of under- or over-expression of UNG on the cellular response to DNA damaging agents.

REFERENCES

1. Kavli B. et al. *J Biol Chem* 2002;277(42):39926-36.
2. Otterlei M. et al. *Embo J* 1999;18(13):3834-44.
3. Fortini P. et al. *J Biol Chem* 1999;274(21):15230-6.
4. Yan L. et al. *Clin Cancer Res* 2007; 13(5):1532-9
5. Tentori L. et al. *Leukemia* 1999;13(6):901-9.
6. Fischer J.A. et al. *Cancer Res* 2006;66(17):8829-37.

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