CHARACTERIZATION OF SYNERGISTIC EFFECT OF IODODEOXYURIDINE AND CLOFARABINE IN CISPLATIN-RESISTANT OVARIAN CANCER CELLS

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ANAND PRAKASH

CLINICAL NUTRITION

ABSTRACT

Epithelial ovarian cancer (EOC) is the most common gynecological cancer and the fifth most frequent cause of cancer deaths in women. Cytoreductive surgery (CRS) followed by platinum-based chemotherapy is used for most of the EOC patients. The main concern in the treatment of EOC is drug-resistance. Many approaches such as new drugs and drug combinations have been used to overcome drug resistance. However, there is limited success in clinic. Therefore there is an urgent need to develop novel combination therapies to improve the clinical outcome of EOC.

In this study we demonstrate that a novel chemo-combination of clofarabine, a purine nucleoside analogue, and iodo-deoxyuridine (IUdR), a thymidine analogue, can be potentially used in cisplatin-resistant ovarian cancers. To evaluate this combination and elucidate the mechanism of chemosensitization of clofarabine by IUdR, we tested two ovarian cancer cell lines SKOV-3 and OVCAR-5, both of which are known to be cisplatin resistant. We have shown that clofarabine has significantly higher cytotoxic effect towards SKOV-3 cells. We also found that IUdR can increase the activity of deoxycytidine kinase (dCK), an enzyme required for the cytotoxicity of clofarabine. Therefore, we hypothesized that clofarabine-IUdR combination will achieve superior
antitumor activity in cisplatin-resistant cancer cells. Our results demonstrate that the combination of clofarabine and IUdR has a synergistic effect in SKOV-3 cells. However, we did not observe similar effect in OVCAR-5 cells. Further investigation revealed that these two cell lines differ in their mismatch repair protein hMLH1 status. While the SKOV-3 cells are hMLH1 deficient, OVCAR-5 cells were hMLH1 proficient. Therefore we tested if the mismatch repair status of the two ovarian cancer cells is playing a role in differential cytotoxic profile. Using siRNA-MLH1, we knocked down hMLH1 expression in OVCAR-5 cells. However, we did not observe that lowered hMLH1 causes dCK activation and clofarabine-IUdR synergism, suggesting that hMLH1 may not play a role in regulation of dCK activation.

In summary, we demonstrate that the novel combination of clofarabine and IUdR can be potentially used in the treatment of some of the ovarian cancers.
DEDICATION

I dedicate this thesis first and foremost to my wife Abhilasha, for her unwavering support and encouragement. Without you I could not have realized my dreams. Second, to my parents who directed me at times, but mostly encouraged me to “figure it out” on my own. Their high hopes for me have motivated me to be the best that I could be and to always strive for more. I hope that you are proud of what I have accomplished and I pray that I can instill similar values in my children. Third, to my younger brothers Arvind and Ashutosh. I wish them the very best in their future lives. And finally, to my daughter Anisha, who is a perfect example of appreciation, happiness and unconditional love.
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And last but not the least I would like to thank my other lab members Xiaoli Cui ‘Lily”, Chunying Yang, Ashish Dutta Dwary, Evan Comeaux and to my friends Simerpreet Singh and Gaurav Nayyar.
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<td>EOC</td>
<td>Epithelial ovarian cancer</td>
</tr>
<tr>
<td>CRS</td>
<td>Cytoreductive surgery</td>
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<tr>
<td>dCK</td>
<td>Deoxycytidine kinase</td>
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<td>hMLH-1</td>
<td>MutL homolog 1</td>
</tr>
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<td>IUdR</td>
<td>5'-Iodo-deoxyuridine</td>
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<tr>
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<td>Thymidine tri phosphate</td>
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<tr>
<td>UTP</td>
<td>Uridine tri phosphate</td>
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<td>MMR</td>
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<tr>
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<td>Cytidine triphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>IR</td>
<td>Ionizing radiation</td>
</tr>
<tr>
<td>MTS</td>
<td>(3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), in phenazine methosulfate (PMS)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>DMSO</td>
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<td>IV</td>
<td>intravenous</td>
</tr>
<tr>
<td>IP</td>
<td>intraperitoneal</td>
</tr>
<tr>
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<td>Super oxide dismutase-1</td>
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<td>base excision repair</td>
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<td>single stranded break</td>
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INTRODUCTION

Overview of Epithelial Ovarian Cancer

Ovarian cancer is the fifth most frequent cause of cancer death in women and about 50% of all cases occurring in women older than 65 years. Epithelial variant of the ovarian carcinoma is one of the most common gynecologic malignancies. Epithelial ovarian cancer (EOC) affects about 204,000 women and is responsible for over 125,000 deaths annually.\(^1\) In the year 2005, there were approximately 174,236 women alive who had a history of cancer of the ovary in the United States. There are an estimated 21,650 new cases and 15,520 deaths due to ovarian cancer in the year 2008 in the US.\(^2\)

Frontline treatment modality used in the treatment of EOC includes cytoreductive surgery (CRS) and platinum-based combination chemotherapy.\(^3\) The rationale behind the use of the CRS followed by combination chemotherapy is to remove the bulk of the tumor which contains poorly oxygenated, non-proliferating cells which might already be chemoresistant or have potential to become chemoresistant, leaving a smaller tumor size with proliferating cells that may be more susceptible to chemotherapy.\(^4\) But about 60% of the patients who
have advanced disease at presentation will need to undergo second look surgery in order to remove any residual tumor.\textsuperscript{5} There are evidences in the literature that the CRS is most effective when there is no macroscopic residual tumor. Even after the CRS, most patients will need postoperative chemotherapy to eradicate the residual microscopic tumor.\textsuperscript{6}

\textit{Chemotherapy and Chemoresistance in Ovarian Cancer}

Patients with EOC usually need to undergo the postoperative chemotherapy to kill metastatic tumor cells because most of EOC patients are diagnosed at advanced stages (stage 3 and stage 4) of the disease. Patients in the early stages of the disease however, will not need chemotherapy because CRS alone has >90\% survival rate in these patients, and chemotherapy in stage 1a or 1b has not been shown to increase the survival in these subgroups of patients.\textsuperscript{7,8} Patients in stage 1c and stage 2, on the other hand will need platinum-based chemotherapy in order to reduce the chances of relapse. These subgroups of patients have shown 80\% disease free survival after the postoperative chemotherapy. Studies have shown that postoperative chemotherapy has survival advantage in this subgroup of patients and that is why there is usefulness of postoperative chemotherapy.\textsuperscript{8,9}

The patient who belongs to the high risk category such as patients with Lynch syndrome, BRCA-1 and BRCA-2 positive status and those who have familial history of breast or ovarian cancer, paclitaxel and carboplatin are used in the early stage of the disease. Although clinical trials have not determined how many cycles of paclitaxel/carboplatin should be used, evidence has shown that lesser cycles of the treatment has higher rate of relapses.\textsuperscript{10,11}
In advanced stages of EOC, primary CRS followed by platinum and taxane based chemotherapy is invariably used. The combination of paclitaxel and the cisplatin analogue, carboplatin, has been shown to increase the progression free disease and overall survival rate in two different clinical trials.\textsuperscript{12,13} Patients in whom the CRS was unable to optimally debulk the tumor to < 1 cm, when treated with paclitaxel and cisplatin chemotherapy, have shown 37 months greater overall survival than those treated with cyclophosphamide and cisplatin combination chemotherapeutic regimen.\textsuperscript{12} Studies have shown that with the addition of paclitaxel as the first line chemotherapy, there was about 30\% reduction in the risk of death.\textsuperscript{12,14}

Combinations of carboplatin with taxane have shown to be equally efficacious with much fewer untoward side effects of chemotherapy as compared to cisplatin and taxane.\textsuperscript{15,16} In patients with advanced disease who have undergone optimal cytoreduction, the cisplatin-paclitaxel combination chemotherapy has been shown to increase the survival by 5 years. However, even after initial success and increase in the median survival, the majority of patients relapse and a second-look surgery will be needed.\textsuperscript{15} About 20\%-30\% of the patients never achieve remission even after cytoreduction and first-line chemotherapy, and the disease continues to progress.\textsuperscript{17}

Recurrence of EOC is a major problem and generally this is the stage when the disease is considered to be incurable and only palliative treatment is offered to relieve the patient’s symptoms. Increase in the levels of ovarian tumor marker CA-125 is a common sign observed in cases of relapse well before any evidence of
clinically significant disease.\textsuperscript{18} This isolated increase in CA-125 levels without clinically significant tumor is called marker-only relapse and it will result in clinical development of recurrence within the median time of 3 months.\textsuperscript{19} Treatment with hormonal drugs has been suggested in such a situation.\textsuperscript{20,21}

Marker-only relapse patients will finally need to be treated with second line chemotherapeutic agents. The choice of second line chemotherapeutic agent/agents depends on the duration of remission after initial first line chemotherapy. Those who have relapsed after 6 months of platinum based regimen are most likely to have platinum sensitive disease and to respond to the subsequent platinum based regimen with response rate $>30\%$.\textsuperscript{22,23} Single agent is used in mild disease in these patients and combination chemotherapy is preferred in those with severe symptoms and rapidly progressive disease. Tumor cytoreduction ($2^{\text{nd}}$ look surgery) is another option for patients who relapse after long first remission ($>6$-$12$ months) before employing the chemotherapy regimen.\textsuperscript{24}

Patients with short remission after first line chemotherapy usually have a platinum-resistance disease and should be treated with regimens without platinum.\textsuperscript{25} The drugs which can be used in suspected cisplatin resistance include liposomal doxorubicin, topotecan, gemcitabine, paclitaxel, etoposide and vinorabine.\textsuperscript{22-26}

The main clinical concern in treatment of ovarian cancer is the development of drug resistance. As many as 75\% of patients with advanced stage EOC will respond to initial (platinum containing) chemotherapy but the majority will relapse within 2 years of completing treatment. To begin with about 25\% of patients are
intrinsically resistant, i.e., will not respond to chemotherapy at all. Usually the shorter the interval from the last platinum chemotherapy to relapse, there is much greater chance that subsequent courses of platinum based chemotherapy will be ineffective. Patients in whom the disease relapses within 6 months of completing treatment have a less than 10% chance of responding to subsequent conventional platinum chemotherapy. Clinical recurrences of the disease that take place within 6 months of completion of a platinum-containing regimen are considered platinum-refractory or platinum-resistant recurrences.

Mechanism of Chemoresistance

Cisplatin reacts with guanine at the N7 position to form platinum-DNA adduct and prevents normal DNA functioning. Resistance to cisplatin develops in two ways, first by limiting the formation of platinum-DNA adduct and second by either decreasing the intracellular drug concentration or increasing the drug inactivation. Cisplatin accumulation in cells takes place through the passive diffusion or facilitated transport. In most cell lines, resistance to cisplatin develop due to decrease in uptake of the drug. Pre-clinical studies show that abnormalities in drug metabolism, accumulation, DNA repair mechanism, cell type and pathways of cell survival are main factors contributing to the development of resistance in ovarian cancer.

An ovarian tumor has been classified as having two types of chemoresistance. De-novo resistance- about 20% of the ovarian cancers will not respond to the initial chemotherapy and are thought to be de-novo resistant. But the majority will initially respond to the chemotherapy but later in the course of the
treatment will acquire resistance. Acquired chemoresistance results due to selection of drug resistant clones while the chemotherapy is ongoing.  

Two factors have been proposed to describe drug resistance in ovarian cancer. A. Pharmacological resistance- even though higher doses of cisplatin are used, a drug resistant population of cells will finally grow out. B. Cellular resistance- which involves multiple mechanisms. DNA damage induced by the various cytotoxic drugs and the repair capability are the main factors which causes the cell to become sensitive or resistant. Many genes are association with micro satellite instability which indicate defect in mismatch repair status has also been found to be associated with the development of cisplatin resistance. Attempts to assign a gene signature to ovarian cancer resistance to chemotherapy have been unsuccessful so far because the development of resistance has been considered as a secondary phenomenon and not the primary response. The development of cisplatin resistance in ovarian cancer has a multi-factorial origin and results from accumulation of multiple genetic changes and resulting in the drug resistant phenotype. Multidrug Resistance-associated proteins have not been found to influence the cisplatin resistance. Glutathione can cause inactivation of cisplatin and can lead to elimination of the drug from the cell. Inactivation of platinum drugs have also been proposed to be due to metallothionine proteins. These proteins play a role in heavy metal detoxification and it has been shown to be associated with cisplatin resistance. Cells possess the capacity to repair DNA damage and this determines the cells sensitivity to the platinum drugs. To inhibit
cells capability to repair the DNA damage is one of the strategies to overcome the cisplatin resistance. 29

Platinum analogues cisplatin and carboplatin are the most effective drugs against ovarian cancer. 36 In a trial based on using single drug treatment, cisplatin was found to be better than the cyclophosphamide. 37 Many major clinical trials have established cisplatin combination chemotherapy as a standard treatment regimen for EOC. 29 Treatment with cisplatin or platinum containing combinations have been found to have much better survival advantage according to a meta-analysis of 45 clinical trials which included more than 8000 patients. Taxanes are also a preferred choice of drugs in combination with cisplatin. A study by the Gynecological Oncology Group showed that after cytoreductive surgery a combination treatment with paclitaxel and cisplatin had better median survival. Carboplatin is preferred over cisplatin because of the lesser side effects. 38-44 No differences have been shown between cisplatin/carboplatin and docetaxel in efficacy of treatment. 45 Currently paclitaxel/docetaxel with either cisplatin or carboplatin is the preferred combination chemotherapy after CRS in EOC patients. 45, 46

The tolerance to platinum-DNA damage is a phenotype that has been found in both acquired as well as in de-novo resistant ovarian cancer. Cells which have lost the mismatch repair (MMR) system have been shown to have a low level of platinum resistance as they remove any mismatch occurring during new DNA synthesis. 29 47 The repair system involved is the nucleotide excision repair (NER) which excise the platinum-DNA adducts leading to homology directed DNA repair
(HP) for precise repair of the DNA. Cisplatin resistance has been proposed to be due to decreased accumulation of cisplatin, increased intracellular trapping of the cisplatin, and increased repair of DNA damage or development of tolerance of DNA damage. Besides these phenomena, many other factors have been reported. 48

In a systematic review of platinum and taxane resistance it has been shown that 68.1% of cisplatin resistant cells were paclitaxel sensitive and 66.7% paclitaxel resistant cells were cisplatin sensitive. This association was not specific to cancer type, agent used or the mechanism of development of resistance. Therefore, it has been suggested that basic understanding of the underlying mechanism of development of resistance, whether cellular or molecular, would be very helpful in identifying new therapeutic targets and also in predicting the response to salvage chemotherapeutic regimens. 49

*Methods to Overcome Chemoresistance in EOC*

Many methods have been developed to treat EOC- either to develop a novel method to increase the cisplatin activity or new drug or drug combinations to increase the cytotoxicity on EOC. Several drugs have been used to enhance the cytotoxicity of cisplatin such as nucleoside analogue gemcitabine, fludarabine and cytarabine, ribonucleotide reductase inhibitor hydroxyurea and DNA polymerase alpha and gamma inhibitor ophidocelin. 29

It has been shown that the different types of the nitric oxides (NO’s) play a novel role in development of cisplatin resistance in ovarian cancer cells. Cisplatin regulates these NO’s differently in cisplatin resistant and cisplatin sensitive ovarian
cancer cells which may be responsible for development of chemo-resistance and inhibition of all NO’s in cisplatin resistant cells resulting in dramatic sensitization and induction of apoptosis.\textsuperscript{50}

Another way to increase chemosensitivity is to use high doses of progesterone. High doses of progesterone can increase cisplatin toxicity in EOC. It causes increased intracellular accumulation and can induce drug toxicity in two cisplatin resistant cell lines (SKOV-3 and OVCAR-3) tested. \textsuperscript{51} Arsenic compounds have also been shown in combination chemotherapy to overcome cisplatin resistance.\textsuperscript{29} Also the combination of intraperitoneal cisplatin therapy with hyperthermia has been shown to achieve the deeper penetration of the drug. \textsuperscript{52}

To overcome resistance, many drugs have been approved by the FDA, such as pegylated liposomal doxorubicin, topotecan and hexamethylamine. Some other drugs such as gemcitabine, oral etoposide, docetaxel and oxaliplatin have been used either in addition to first line chemotherapy or as second line drugs. Currently strategies are being explored for more selective delivery of drugs such as intraperitoneal or radio-labeled monoclonal antibodies, to target drug resistance by targeting specific protein/ receptor and tumor vasculature. Other strategies such as agents to inhibit PI-3 kinase, mTOR protein and K-ras may be of a potential therapeutic benefit in the future.\textsuperscript{53} In a study, selenite/selenothionine compounds have been shown to prevent the development of drug resistance in ovarian carcinoma xenograft models. \textsuperscript{54}

Intraperitoneal (IP) chemotherapy has been shown in many clinical trials to have much better clinical response because of the better drug delivery at the
diseased site. This route was designed in order to maximize the efficacy of the drug and reduce its systemic side toxicity. There was significant increase in the peritoneal cavity drug concentration and exposure by IP route when compared to the systemic vascular compartment. A study by gynecological oncology group which compared the intravenous (IV) and IP route of chemotherapy showed that there was significant increase in survival in the patients with EOC receiving IP from 49 months to 66 months. According to the new National Cancer Institute recommendations IP route should be offered to these patients for the frontline treatment of ovarian cancer.

Some methods have been introduced to increase the intracellular concentration of cisplatin. Hyperthermia has been demonstrated to increase the cytotoxicity. Superoxide dismutase-1 (SOD-1) has been the potential target to overcome the cisplatin resistance in human ovarian cancer and it has been proposed that modulation of SOD-1 activity may be helpful in overcoming the resistance. Another method is the development of non-cross-resistant platinum based drugs which are cytotoxic even in the presence of cisplatin resistance.

Development of drugs and other techniques to decrease the drug resistance to cisplatin and elucidating the mechanism will be more helpful in predicting the clinical response and may identify novel mechanism-based new treatment options to improve the patient outcome.
Nucleoside Analogue Clofarabine and Deoxycytidine Kinase (dCK)

**Clofarabine**

Clofarabine, a nucleoside analogue, was developed at Southern Research Institute, Birmingham, Alabama. Clofarabine received the US-FDA approval in 2004 for relapsed or refractory acute lymphoblastic leukemia patients of the pediatric age group 1-21 years who had undergone treatment with at least two prior regimens. Clofarabine is converted to its monophosphate form by deoxycytidine kinase (dCK). Then monophosphate and diphosphate kinase converts clofarabine into its active form clofarabine tri-phosphate. Clofarabine-triphosphate halts DNA synthesis by inhibiting ribonucleotide reductase and DNA polymerase. This active form of clofarabine has cytotoxic effects on quiescent as well as proliferating cancer cells. Both preclinical and clinical trials have shown that clofarabine is active against leukemia and many solid tumors including breast cancer, colorectal cancer and prostate cancer. Clofarabine is also known to act as a radio-sensitizing agent both, *in vivo* and *in vitro* by its ability to interfere with DNA damage responses.

**Deoxycytidine kinase (dCK)**

dCK is a cytosolic protein of molecular weight ≈31 KDa which when over-expressed, mainly localizes in the nucleus. Resistance to nucleoside analogues has been found to be linked to dCK deficiency. dCK expression can be used as a predictor for initial response to nucleoside analogue and final treatment outcome. Activation of dCK occurs by phosphorylation of dCK at Thr-3, Ser-11, Ser-15, and Ser-74 residues. It have been demonstrated that out of these four
sites Ser-74 is the major site of phosphorylation and most important for dCK activity.\textsuperscript{67}

It has been shown that there is enhancement in dCK activity after cells were treated with different types of inhibitors of DNA synthesis. Thus the enhancement of dCK activity by various DNA synthesis inhibitors has been suggested as a rationale for its use in combination chemotherapy.\textsuperscript{68} 2'-deoxythymidine-5'-thiosulphate (dThd-5'TS), a derivative of thymidine is known to increase the dCK activity.\textsuperscript{69} γ-radiation has also been shown to increase the dCK activity.\textsuperscript{70} It has been suggested that dCK undergoes conformational changes during its activation process resulting in a catalytically more active structure.\textsuperscript{71} Activation of dCK following DNA damage and during the repair process suggests its role in the repair process as well.\textsuperscript{70}

**Thymidine Analogue 5'-Iodo-Deoxyuridine (IUdR)**

5'-Iodo-deoxyuridine (IUdR) is a nucleoside and thymidine analogue. IUdR gets phosphorylated to 5'-ido-dUTP and then competes with TTP for incorporation into the DNA.\textsuperscript{72} IUdR has been shown to target mismatch repair deficient (MMR-/−) human cancer cells selectively and causes an increase in radiation-induced cytotoxic damage (radio-sensitization).\textsuperscript{73} IUdR can increase the radio-sensitization of MMR-/− cells. It has been shown that MMR status determines the cytotoxicity and IUdR-DNA incorporation in human colon cancer cells. MMR-/− cells were more sensitive than mismatch repair proficient (MMR+/+) cells.\textsuperscript{63} MMR deficiency imparts resistance to commonly used chemotherapeutic agents. IUdR
has been shown to selectively target such tumor cells and also enhance the radio- 
sensitivity. 74

Cytotoxic effect of IUdR and radio-sensitization is affected by IUdR-DNA 
incorporation, DNA single stranded break (SSB) and double stranded break (DSB) 
causing cell death. Base excision repair (BER) is a known major pathway for SSB 
by either chemical agents or ionizing radiation(IR), thus impairment of BER has 
been shown to increase IUdR related cytotoxic effect and radiation sensitivity in 
mammalian cells.75 Also IUdR can be used in both hMLH-1 and MSH-2 deficient 
cells which are resistant to radiation treatment. Halogenated thymidine analogues 
are able to selectively target mismatch deficient cells and it has been suggested 
that IUdR can be used in the drug resistant cells that lack both hMLH-1 and 
MSH2.76 It is known that loss of MMR activity is responsible for cisplatin resistance 
and cisplatin treatment causes differential p53 accumulation in these types of cells. 
P53 imparts a protection to cisplatin induced DNA damage in the cells lacking 
hMLH1 in human colon cancer cell HCT116.77 hMLH-1 deficient state is 
responsible for causing IUdR induced radio-sensitization.78 Because dCK is an 
integral step in activation of clofarabine and thymidine is known to increase the 
dCK activity, we hypothesized that thymidine analogue IUdR can increase the dCK 
activity and thus the cytotoxicity of clofarabine. IUdR is a known radio sensitizer, 
and in our study we will show that it can also be used as a chemo-sensitizer for 
nucleoside analogue clofarabine.
Mismatch Repair Protein hMLH-1 and Drug Resistance

hMLH-1 is a nuclear localized protein. It is also known as COCA2, FCC2, and HNPCC2. The protein consists of 756 amino acids with a molecular weight of 84.6 KDa. The hMLH-1 protein is involved in the repairing process of mismatched bases during DNA replication. hMLH-1 has two interaction domains and one ATPase domain. Of the two interaction domains one is for MutS homologue and the other one is for PMS2, MLH3 or PMS-1. hMLH-1 does not have any known enzymatic activity. It forms a heterodimer with PMS-2 called Mut La. This heterodimer then forms another complex with heteroduplex Mut Sa, which has the ability to recognize the DNA lesions. The hMLH-1 heterodimer then recruits the proteins responsible for excision and repair of the DNA.

The role of hMLH-1 has been implicated in predisposition to develop certain cancers, such as colorectal, endometrium, ovary, small intestine, biliary tract, brain, urinary tract and stomach. hMLH-1 mutation causes loss of heterogeneity that leads to mismatch repair deficient status and thus accumulation of mutations that causes micro satellite instability and promotes tumorogenesis. Another entity with which it is associated is the microsatellite instability. The molecular feature which leads to tumorogenesis is loss of MMR system.  

Summary

Cisplatin-resistance in ovarian cancer cells either at the beginning or during the later stage of chemotherapy is the major clinical concern in the treatment of ovarian cancer. Cisplatin is known to be the most effective drug in ovarian cancer
treatment but a majority of patients will eventually develop resistance to cisplatin. We proposed that a new drug combination of clofarabine and lodo-deoxyuridine will have much better cytotoxicity in ovarian cancer cells that show cisplatin resistance. In this study we will elucidate the role of our new drug combination, the molecular mechanism of action and how mismatch repair protein hMLH-1 plays a role, if any, in relation to the dCK activation process which is an integral step in the mechanism of action of nucleoside analogue clofarabine.
CHAPTER 2

A NOVEL COMBINATION TREATMENT FOR CISPLATIN-RESISTANT OVARIAN CANCER

Enhanced Cytotoxic Effect of Clofarabine in Cisplatin-Resistant SKOV-3 Ovarian Cancer Cells

In order to find new approaches to improving the treatment outcome of chemo-resistant ovarian cancer, we tested the anti-tumor activity of clofarabine, a newly developed nucleoside analogue. Clofarabine has been shown to have superior cytotoxicity to many solid tumors, therefore it was reasonable to test its activity against ovarian cancer. First we compared the cytotoxic effect of clofarabine with cisplatin in two ovarian cancer cell lines (SKOV3 and OVCAR-5). SKOV-3 and OVCAR-5 are known to be cisplatin-resistant. Clonogenic assays were performed and the data demonstrated that clofarabine displayed significantly greater cytotoxicity in SKOV-3 cells in a dose dependent manner (Figure 1A). The cytotoxic effect of clofarabine is much higher as compared to the cisplatin at similar doses. Because OVCAR-5 cells did not form colonies in our clonogenic assay, we performed the MTS cell cytotoxicity assay to assess the cytotoxic effect of clofarabine on these cells. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), forms water soluble formazan by living cells which is then read at 490nm by colorimeter in the
presence of phenazine methosulfate (PMS). Figure 1B showed the enhanced cytotoxicity of clofarabine at 1000 nano molar dose in SKOV-3 cells, similar to what we have observed in the clonogenic assay which was statistically significant, although we did not see a significant cytotoxicity at lower doses. However, the OVCAR-5 cells showed significant difference statistically between the cytotoxicity of cisplatin versus clofarabine at various doses (Figure 1C), there was very little enhancement of cytotoxic effect. Therefore, these observations suggest that clofarabine has higher cytotoxicity in cisplatin-resistant ovarian cancer cells, SKOV-3.
CIS: Cisplatin, CLF: Clofarabine

Figure 1A: Clonogenic assay for SKOV-3 cells. Clonogenic assay with SKOV-3 shows that there is much greater cytotoxicity with clofarabine when compared to the similar doses of the cisplatin in this ovarian cancer cells. Each data point shows the mean and standard deviation of a single experiment performed in triplicate. Clofarabine was significantly greater cytotoxic (p<0.05) as compared to cisplatin.
CIS: Cisplatin, CLF: Clofarabine

Figure 1B: MTS cell cytotoxicity assay for SKOV-3. Clofarabine has better cytotoxicity in SKOV-3 cells as compared to cisplatin. The cell cytotoxicity assay by MTS showed that increasing dose of clofarabine exerts more cytotoxic effect in this cisplatin resistant ovarian cancer cells. Each data point shows the mean and standard deviation of a single experiment performed in triplicate. Clofarabine was significantly greater cytotoxic (p<0.05) as compared to cisplatin at 1000 nano molar dose in MTS cell cytotoxicity assay.
CIS: Cisplatin, CLF: Clofarabine

Figure 1C: Cell cytotoxicity assay (MTS ASSAY) in OVCAR-5 cells. Clofarabine was not able to show the enhanced cytotoxicity in OVCAR-5 ovarian cancer cells. OVCAR-5 cells are known to be mildly cisplatin resistant but clofarabine is unable to show much cytotoxic effect in these cells when compared with SKOV-3 cells. Each data point shows the mean and standard deviation of a single experiment performed in triplicate. Though clofarabine was significantly greater cytotoxic statistically (p<0.05) as compared to cisplatin, there was not enough cell death as compared to the cisplatin-treated group.
IUdR enhances dCK activity in SKOV-3 cells

In order to develop a molecularly-targeted combination therapy for cisplatin-resistant ovarian cancer, we investigated the role of deoxycytidine kinase (dCK), which is required for clofarabine cytotoxicity and hypothesized that if there is an agent that can increase dCK activity, the combination of this reagent with clofarabine would produce a synergistic effect. Another research project led by Dr. Michael Lee in our laboratory investigated the change of dCK activity in a cell cycle dependent manner. The data showed that when HeLa cells were synchronized with thymidine double block, there was a cell cycle dependent statistically significant (p<0.05) increase in the dCK activity as compared to the unsynchronized cells (Figure 2A) after DNA damage. More importantly we observed that dCK activity is significantly increased with thymidine treatment. Therefore we hypothesized that any thymidine analogues might be able to enhance dCK activity in ovarian cancer cells and can work synergistically with clofarabine. To test this hypothesis, we chose IUdR (5'-Iodo-deoxyuridine), a thymidine analogue to observe its effect on dCK activity in SKOV-3 cells. The measurement of dCK activity was conducted in Dr. William B. Parker's laboratory. To measure the dCK activity an indirect method was used. Arabinoside-C (Ara-C) is a substrate for the dCK and we measured the Ara-CTP peaks by High Pressure Liquid Chromatography (HPLC) as an indirect measure of the dCK activity. In the SKOV-3 ovarian cancer cells we observed about 20% increase in dCK activity after 24 hour treatment with 10 µMolar IUdR (Figure 2B).
Therefore it is demonstrated that IUdR can significantly (p<0.05) activate dCK and can be tested as a sensitizing agent with clofarabine.

Ara-CTP/GTP: Indirect measure of dCK activity, Unsyn: Unsynchronised cells, S: S phase of cell cycle, G2: G2 phase of cell cycle, G1: G1 phase of cell cycle

Figure 2A: Following synchronization by double thymidine block HeLa cells were released for 2hrs, 6hrs, or 12hrs Corresponding to G1, S, and G2 phases of the cell cycle and then Subsequently treated 100μmolar Ara-C for 30 min which serves as a substrate for dCK. There was significantly greater dCK activity as compared to the unsynchronized cells in all the phases of cell cycle. (p<0.05) Each data point shows the mean and standard deviation of a single experiment performed in triplicate.
Ara-CTP/GTP: Indirect measure of dCK activity, DMSO: control treatment, IUdR: 10 µmolar IUdR treatment

Figure 2B: dCK activity following IUdR treatment in SKOV-3 cells. SKOV-3 cells following 10 µmolar IUdR treatment for 24 hrs showed increase in the dCK activity as measure by Ara CTP/GTP ratio. Ara-c is the substrate for the deoxycytidine kinase and increase in the Ara CTP/GTP ratio is an indirect measure of the dCK activity. Each data point shows the mean and standard deviation of three separate experiments. The p value for the two groups was 0.05.
Clofarabine-IUdR Combination Produces Synergistic Effect on Anticancer Activity

First we determined if IUdR itself has cytotoxic effect in SKOV-3 cells. Since IUdR can increase dCK activity, we further tested whether there is any synergistic effect on anticancer activity using Clofarabine- IUdR combination. As figure 3A shows there was significant difference with 2 micromolar IUdR treatments compared to untreated cells but no difference has been found with 4 micromolar treatments when compared to the untreated group. To achieve this goal, we performed the clonogenic assay in SKOV-3 cells treated with the drugs. Figure 3B showed that 2 micro molar of IUdR treatment significantly increased clofarabine cytotoxicity. As the dose of IUdR increases to 4 micro molar the synergistic effect is also enhanced. Addition of IUdR to clofarabine further decreased the cell survival from 30% to less than 20% in the colony formation assay and from 40% to less than 30% in the MTS cell cytotoxicity assay. It is noted that the IUdR doses we used for the experiments have limited cell toxicity by itself; therefore the combinational effect is considered synergistic. These findings were further confirmed by MTS assay (Figure 3C).

We also attempted to test whether there is synergistic effect of clofarabine and IUdR combination in OVCAR-5 cells. However, we were unable to observe similar effects using the MTS assay. As the OVCAR-5 cell line was derived from untreated ovarian carcinoma (advance stage) patient and had cisplatin resistance in vitro (low level of resistance).80 This cell line does not show enhanced cytotoxicity by clofarabine alone or with the addition of IUdR (data not shown).
Summary

Our results demonstrate that clofarabine has anti-tumor activity in cisplatin-resistant ovarian cancer cells. Further we demonstrate that IUdR, a thymidine analogue can sensitize clofarabine activity by enhancing dCK activity.

Figure 3A: Dose response of IUdR on SK0V-3 cells. 2μmolar of IUdR treatment showed statistically significant difference (p<0.05) than untreated group but 4μmolar dose was not statistically different than the untreated group.
CIS: Cisplatin, CLF: Clofarabine, CLF+2µMolIUdR: varying doses of clofarabine with fixed dose of 2µm of IUdR, CLF+4µMol IUdR: varying doses of clofarabine with fixed dose of 4µmolar of IUdR.

Figure 3B: Clonogenic assay for SKOV-3 cells following drug treatment. Following treatment of SKOV-3 cells with varying doses of cisplatin, clofarabine and combination of clofarabine with either 2µmol or 4 µmol of IUdR shows that these cells have little if any response to the cisplatin but clofarabine alone has better cytotoxicity as compared with cisplatin. When clofarabine was combined with IUdR there was increased cytotoxic effect. Keeping the dose of IUdR constant and increasing dose of clofarabine yielded enhanced cytotoxicity as IUdR dose increased from 2 µmol to 4 µmol. Each data point shows the mean and standard deviation of a single experiment performed in triplicate. The used doses and the cytotoxicity of clofarabine alone and clofarabine-IUdR combination had statistically significant cytotoxic effect as compared to cisplatin. (p<0.05)
CIS: Cisplatin, CLF: Clofarabine, CLF+2 µmol IUdR: varying doses of clofarabine with fixed dose of 2 µmol of IUdR, CLF+4 µmol IUdR: varying doses of clofarabine with fixed dose of 4 µmol of IUdR.

Figure 3C: MTS cell cytotoxicity assay for SKOV-3 cells. Similar to the colony formation assay MTS assay showed that SKOV-3 cells have little if any response to the cisplatin but clofarabine alone has better cytotoxicity than cisplatin. When clofarabine was combined with IUdR there was increased cytotoxic effect. Keeping the dose of IUdR constant and increasing dose of clofarabine yielded enhanced cytotoxicity. We get even more enhancement of cytotoxic effect when the dose of IUdR was increased from 2 µmol to 4 µmol. Each data point shows the mean and standard deviation of a single experiment performed in triplicate. The used doses and the cytotoxicity of clofarabine alone and clofarabine-IUdR combination had statistically significant cytotoxic effect as compared to cisplatin. (p<0.05)
CHAPTER 3

ROLE OF THE MISMATCH REPAIR PROTEIN hMLH-1 IN REGULATION OF DCK ACTIVATION

hMLH-1 Proficiency in Ovarian Cells is Associated With Lack of IUdR-Induced dCK Activation

Our experiments have demonstrated that IUdR can activate dCK in SKOV-3 cells (Figure 2B). However, it was also observed that IUdR failed to enhance dCK in OVCAR-5 cells (Figure 4), suggesting that IUdR-induced dCK activation is regulated by an unknown factor that differs in these cell lines. We performed a literature search and learned that as compared to OVCAR-5, SKOV-3 is mismatch repair deficient (hMLH-1). To confirm this, we conducted western blot analysis using an anti-MLH1 antibody on total cell lysates from these cells, and we found that SKOV-3 cells showed no expression of hMLH-1 when compared to OVCAR-5 cells (Figure 5).

hMLH-1 siRNA Mediated Knockdown Enhances IUdR-Clofarabine Cytotoxicity in OVCAR-5 Cells

To demonstrate the role of hMLH-1 in IUdR-induced dCK activation, we knocked-down hMLH-1 in OVCAR-5 cells using the SiRNA approach. Transfection of siRNA was accomplished by Nucleofection (see material and
methods) OVCAR-5 cells transfected with hMLH-1 SiRNA showed lowered hMLH-1 expression as compared to control-siRNA transfected cells (Figure 6A). We confirmed the western blot signal by densitometry and as shown in Figure 6B there was 50% knockdown in hMLH-1 expression when compared to the control siRNA transfected OVCAR-5 cells. Because we could not get repeated results with the used method of knockdown, we could not perform the statistical analysis if the knockdown was statistically significant. Further we attempted to measure dCK activity after the 10 micro molar of IUdR treatment for 24 hours. This would have allowed defining the role of hMLH-1 in dCK regulation. However, we failed to obtain the data because of technical difficulties in the HPLC assay using transfected cells. After transfection with either hMLH-1 SiRNA or control SiRNA, we were unable to get any peaks for Ara-CTP by HPLC analysis of the samples. Therefore we could not measure the dCK activity in OVCAR-5 cells after transfection (data not shown). Inability to obtain Ara-CTP peaks in the HPLC analysis of the OVCAR-5 transfected cells has many potential explanations. It might be possible that the transfection reagent used is interfering with the Ara-CTP peaks because we could not obtain the peaks for Ara-CTP in any our samples, though we were getting Ara-CTP peaks in the non-transfected OVCAR-5 cells.
DMSO: control treatment, Ara-CTP/GTP: indirect measure of dCK activity

Figure 4: dCK activity following IUdR treatment in OVCAR-5 cells. OVCAR-5 cells following 10μmolar IUdR treatment for 24 hrs showed increase in the dCK activity as measure by Ara CTP/GTP ratio. Ara-c is the substrate for the deoxycytidine kinase and increase in the Ara CTP/GTP ratio is an indirect measure for the increase in the dCK activity. Each data point shows the mean and standard deviation of a single experiment performed in triplicate. 24 hour treatment with 10 μ molar IUdR failed to enhance the dCK activity in OVCAR-5 cells (p=0.40)
Figure 5: Western blot showing the MLH-1 expression in OVCAR-5 and SKOV-3 cells. OVCAR-5 express hMLH-1 but the SKOV-3 cell does not.

Figure 6A: Western blot of OVCAR-5 cells after transfection with hMLH-1 SiRNA and control SiRNA-A. There was hMLH-1 knockdown in OVCAR-5 cells after hMLH-1 SiRNA transfection as compared to the control siRNA.
Figure 6B: Quantification of the hMLH-1 versus Control siRNA knockdown signals by densitometry. As we could only show knockdown only once, we could not do a statistical analysis. But this graph is showing 50% knockdown by hMLH-1 siRNA when compared to the control siRNA.

hMLH-1 Does Not Play a Role on Clofarabine-IUdR Combination Treatment

To explore whether hMLH-1 knock-down would lead to an increased synergistic effect of clofarabine-IUdR combination, we performed MTS assay using the hMLH-1 siRNA and control siRNA transfected OVCAR-5 cells. As shown in Figure 7A and 7B, we can see from there is similar cytotoxicity effect
after the hMLH-1 knockdown when compared to the control siRNA transfected OVCAR-5 cells treated with the combination regimen. Though in both control and hMLH-1 group there was statistically significant greater cytotoxicity but it does not appear to have enhanced the cytotoxic effect of clofarabine and IUdR combination to draw any concrete conclusion about the effect of combination treatment. Therefore, it appeared that hMLH-1 does not play a role in clofarabine-IUdR combination induced cytotoxicity.

Summary and Limitations

Our original hypothesis was that hMLH-1 plays a critical role in dCK activation induced by IUdR, and that hMLH-1 knock-down would increase the synergistic effect of clofarabine-IUdR treatment. While with our experiments we confirmed that OVCAR-5 cells expresses hMLH-1 and were able to knock-down hMLH-1 in OVCAR5 cells using siRNA by 50%, we were unable to detect any dCK activity in both control SiRNA as well as hMLH-1 SiRNA transfected OVCAR-5 cells. Further, when we attempted to observe the role of hMLH-1 on clofarabine-IUdR combination, we found that the hMLH-1 status had limited effects on the synergisms of clofarabine-IUdR combination treatment. Even though there is statistically significant increase in cytotoxicity in both control and hMLH-1 transfected OVCAR-5 cells, there is not enough increase in cytotoxic effect. As our siRNA knockdown experiment does not show the complete knockdown of hMLH-1 and we could not repeat the similar results it is very difficult to draw any conclusion. Thus because of these technical difficulties it is
very difficult to say if hMLH-1 plays a role in the cytotoxic effect of clofarabine-IUdR combination treatment.

CIS: Cisplatin, CLF: Clofarabine, CLF+2UM IUdR: varying doses of clofarabine with fixed dose of 2um of IUdR, CLF+4UM IUdR: varying doses of clofarabine with fixed dose of 4um of IUdR.

Figure 7A: MTS assay in hMLH-1 SiRNA transfected OVCAR-5 cells. After hMLH-1 transfection of the OVCAR-5 cells the MTS assay shows that there is indeed enhanced cytotoxicity with clofarabine alone, which was further enhanced by addition of IUdR. Though there is statistically significant (p<0.05) difference in clofarabine and clofarabine-IUdR treatment group, the toxicity was not much enhanced as compared to the control siRNA transfected OVCAR-5 cells. Each data point shows the mean and standard deviation of a single experiment performed in triplicate.
CIS: Cisplatin, CLF: Clofarabine, CLF+2UM IUdR: varying doses of clofarabine with fixed dose of 2um of IUdR, CLF+4UM IUdR: varying doses of clofarabine with fixed dose of 4um of IUdR.

Figure 7B: MTS assay in control SiRNA-A transfected OVCAR-5 cells. After control SiRNA transfection of the OVCAR-5 cells the MTS assay shows that there is indeed enhanced cytotoxicity with clofarabine alone, which was further enhanced by addition of IUdR. Though there is statistically significant (p<0.05) difference in clofarabine and clofarabine-IUdR treatment group, the toxicity was not much enhanced as compared to the cisplatin, the cytotoxic effect was no similar to hMLH-1 siRNA transfected OVCAR-5 cells. Each data point shows the mean and standard deviation of a single experiment performed in triplicate.
CHAPTER 4
MATERIALS AND METHODS

Cell Lines and Media

For our experiments two ovarian cancer cell lines SKOV-3 and OVCAR-5 were used. SKOV-3 cells were purchased from ATCC, Manassas, VA, USA (ATCC no. HTB77). OVCAR-5 cells were a kind gift by Dr William Waud at Southern Research Institute, Birmingham, Alabama. For OVCAR-5 cells, RPMI1640 (Mediatech Inc., VA) was used with 4.5g/L of glucose and L-glutamine without sodium pyruvate. The medium was supplemented with 10% FBS (Fetal Bovine Serum) and 1% Antibiotic (Penicillin-10,000 U/ml, Streptomycin-10,000μg/ml). For SKOV-3 McCoy’s 5A medium (with 1.5 Mm L-glutamine and 2.2 g/L sodium bicarbonate) was purchased from Hyclone, UT. The medium supplemented with 10% FBS (Fetal Bovine Serum) and 1% Antibiotic (Penicillin-10,000 U/ml, Streptomycin-10,000μg/ml). The antibiotics were purchased from Invitrogen. The cells were grown in the serial culture and incubated at 37° C and 5% CO₂. The media was replaced every 3rd day and cells were sub-cultured when they became about 90% confluent.

Colony Formation Assay

Using 6-well culture plates cells were plated as with 200, 400 and 800 cells for 24 hours before they were treated with 0, 10, 50, 100, 500, 1000 nano
molar of cisplatin alone, clofarabine alone or in combination with 2μ molar and 4 μ molar of IUdR. Each treatment was given for 24 hours. After 24 hours the medium was replaced with fresh medium. All plates were incubated in 5% CO₂ and 37°C atmosphere. Colony counting was done on the 14th day of plating the cells using 1x crystal violet solution for staining the colonies. Colonies with greater than 50 cells were taken into account.

**MTS Cell Cytotoxicity Assay**

Using 96-well plate 5000 cells were plated in each well in triplicates. After 24 hours of incubation, the medium was replaced with medium containing the drugs. Drug treatment was given as 0, 50, 100, 500 and 1000 nano molar with cisplatin alone, clofarabine alone and with 2μ molar or 4μ molar IUdR with 0-1000 nano molar of concentration of clofarabine. Drug treatment was given for 72 hours. After this time 20 micro liter of MTS reagent (Cell Titer 96® Aqueous One Solution Cell Proliferation Assay from Promega, Madison, WI catalog no. G3581) was added to each well and the plated were incubated at incubated in 5% CO₂ and 37°C atmosphere in incubator for 4 hours. After the 4 hours period the plated were absorbance was read using a 96 well plate reader by colorimetric method at 490 nanometers.

**dCK Activity Assay**

dCK activity was measured by measuring the Ara-C triphosphate (Ara-CTP) levels by HPLC. This is an indirect method to measure the dCK activity. In short, approximately 1 million cells were plated in T-25 flask for 24 hours. After
24 hours treatment was given with either DMSO, or 10 micro molar of IUdR. Cells were harvested and counted so as to make sure about the same number of cells were in each sample. Cell pellets were suspended in 1 ml of ice cold Puck’s saline, transferred to a microfuge tube, centrifuges and then supernatant removed. Pellet were re-suspended in 200 μ liter of ice cold 0.5M HCLO₄. Mixed and stored on ice for 5 min, centrifuged and 200 ml of acid soluble fraction removed. Then 30micro liter of 1.58 N KOH and 2.5 M of K₃PO₄ solution added, which resulted in a large precipitate. Samples are mixed well and pH is checked to ensure neutral pH. Microfuge tubes were then centrifuged for 5 min and supernatant is removed and stored in -20°C till analyzed by High Performance Liquid Chromatography (HPLC). All the treatment groups were in triplicates so to avoid error because of the cell numbers. Samples will be then analyzed by HPLC in pharmacology Lab at Southern Research Institute. The KCLO₄ precipitate was removed by centrifugation, and a portion of supernatant fluid was injected onto a BioBasic anion exchange high-performance liquid chromatography column (5um; 250x4.5mm Thermo Electron Corp., Bellefonte, PA). Elution of the nucleotides was accomplished with a 30 minutes linear salt and pH gradient (at a flow rate of 1micro liter/min). The initial elution condition were 70% 5 milimolar NH₄H₂PO₄ (pH 2.8) and 30% 750 milimolar NH₄H₂PO₄ (pH 6.0), and the final elution condition were 50% 5mili molar NH₄H₂PO₄ (pH 2.8) and 50 % 750mili molar NH₄H₂PO₄ (pH 6.0). Ultraviolet absorbance was measured at 270 nano meter, which is the UV max for Ara-CTP. This gradient separated the natural nucleotides (UTP, CTP, ATP, and GTP) from one another and from Ara-CTP.
Electrophoresis and Western Blotting

Cell lysates were prepared using the cell lysis buffer. Protein quantification was done by method of Bradford method. 30 μ gram proteins were loaded into the 12.5% SDS gel (Bio-Rad) and ran at 125 volt for 90 minutes. After overnight transfer onto nitro-cellulose membrane, membranes were cut at appropriate band width. The membrane was blocked in 5% milk for 1 hr and then washed with washing buffer 10 min x 3. Then membranes were incubated in the primary antibody for hMLH-1(1:100) (Santacruz biotech, Inc catalog no. sc-56159) and β-tubulin (1:2000). The next day secondary anti-mouse antibodies (1:5000) were added for 1 hr. Membranes washed for 10min x3. Enhance chemiluminiscent (ECL, GE Life Sciences catalog no RPN2106) reagent is added for a minute and membrane is exposed for appropriate time.

siRNA Knockdown of hMLH-1

For transfection of the OVCAR-5 cells nucleofection method was used. Serially growing OVCAR-5 cells were used with 80-90% confluence. For each sample 1 million cells were harvested and 100μliter of nucleofector solution V (Cell Line Nucleofector Kit V Cat. No. VCA-1003, Amaxa, Germany) is added along with either 15 μliter of MLH-1 SiRNA (MLH-1 SiRNA(h) Cat No. sc35943,Santa Cruz Biotech Inc, CA) and 10 microliter of control SiRNA-A(control SiRNA-A Cat No sc37007, Santa cruz Biotech Inc, CA). Then this sample is transferred to cuvette and run with program D-020 in nucleofector for high transfection efficiency. Thereafter cells were transferred to the T-25 flask
with normal growth medium from the Amaxa certified cuvette and put into incubator at 37°C and 5% CO₂ atmosphere. After 24 hours the medium was replaced with fresh medium to remove the dead floating cells. SiRNA knockdown of hMLH-1 was analyzed by western blot. Transfected sample was used either for dCK activity assay or MTS cell cytotoxicity assay.

Statistical Analysis

Data analysis was done by Student t-test and graphs were generated using Microsoft Excel Spread sheet. Mean and standard deviation were calculated either from single experiments that were in triplicate or from three separate experiments. Values of p< 0.05 were taken as statistically significant.
For ovarian cancer patients CRS followed by chemotherapy is the most effective treatment regimen. But chemoresistance is the most important negative factor in the prognosis and survival in patients with EOC. The main reason of failure to respond to the chemotherapeutic regimens is either that the tumor is intrinsically resistant or it develops cisplatin resistant clones during the course of treatment. Many strategies have been employed to overcome the problem of cisplatin resistance, but so far in the clinical setting none of them have proven to be effective enough to enhance the survival of the EOC patients. Our goals are to develop novel approaches of combination treatments to overcome chemoresistance.

In our study we used a novel combination treatment regimen (Clofarabine and IUdR combination). This combination strategy was based on our observations that 1) clofarabine, a newly developed nucleoside analogue has antitumor activity in cisplatin-resistant ovarian cancer cells, 2) clofarabine requires dCK activation, and 3) IUdR, a thymidine analogue can activate dCK. Therefore the combination strategy is molecularly based and has shown synergistic effect on ovarian cancer cells.
To further identify the molecular mechanism governing IUdR-induced dCK activation and identify determinants important for the synergistic effect, we compared two ovarian cancer cells (SKOV-3 and OVCAR-5) which are known to be cisplatin resistant \textit{in vitro}. Interestingly, they showed different cytotoxic profile to clofarabine and clofarabine- IUdR combination treatment. Thus we looked at the available literature which suggested that these two cell lines differ in their mismatch repair protein hMLH-1 status. While the SKOV-3 cells lack hMLH-1, OVCAR-5 cells are hMLH-1 proficient. We confirmed the hMLH-1 status of the two cell lines. Since evidences in literature suggest that hMLH-1 plays a role in development of drug resistance in ovarian and other human cancers, we assessed the effect of hMLH-1 status and differential cytotoxicity to clofarabine- IUdR- combination and dCK activity in these two cell lines. The hMLH-1 siRNA transfection of OVCAR-5 cells exhibited a 50% decrease in the hMLH-1 expression. We then performed the dCK activity assay and cell cytotoxicity assay to elucidate the role of hMLH-1 in dCK regulation. Unfortunately, we were unable to get any peak for Ara-CTP by HPLC analysis in both control siRNA and hMLH-1 siRNA transfected OVCAR-5 cells. One of the explanations is the transfection reagents might have an effect on measuring the Ara-CTP peaks. An alternative method was to look at dCK activation by immunoblotting. DCK activity is dependent on phosphorylation of several sites critical for the enzymatic activity. Among them, Serine 74 is a critical amino acid and it gets phosphorylated when the kinase is active. Therefore measuring Ser74 phosphorylation can also assess dCK activity. We obtained the anti-phospho-Ser74 antibody from Dr. Francois
However, we failed to detect any significant signal using the antibody. One explanation is that there might be cell-type specific dCK activation and in ovarian cancer cells, such as that in SKOV3 and OVCAR-5, the level of dCK activation and Ser74 phosphorylation are low such that the antibody was not sensitive enough to detect the changes.

Nevertheless, the role of hMLH-1 on the synergism of clofarabine-IUdR combination was assessed by MTS assay in OVCAR-5 cells. The data obtained suggest that hMLH-1 might not play a significant role on the synergism since siRNA knockdown hMLH-1 showed limited effect on the synergism. These observations indicate that there might be other factors playing a role on regulation of dCK activation.

Although our presented data in this study are very preliminary and have certain limitation such as inability to get ARA-CTP peaks after transfection, no complete knockdown of hMLH-1, and because most of the experiments were performed only once so we could not perform vigorous statistical analysis. We will perform more experiments to confirm our preliminary findings.

In summary, we have demonstrated a novel combination treatment approach using clofarabine and IUdR. Future studies should be focused on establishment of treatment schedules on the combination treatment and identification of potential factors that might influence the combination effect of clofarabine and IUdR.
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