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Epigenetic Therapies for Chemoresensitization of Epithelial Ovarian Cancer

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Summary

Epigenetic drugs have been shown to enhance gene expression and drug sensitivity in ovarian cancer cell lines and animal models. Based on promising pre-clinical studies, DNA methylation inhibitors in combination with existing chemotherapeutic agents have the potential for overcoming acquired drug resistance, laying the foundation for this specific class of epigenetic drug in ovarian cancer clinical trials. The recent completion of phase I trials of decitabine have yielded important information on dosing schedules and biological endpoints for evaluating patient responses. In addition, epigenetic drug effects on pharmacodynamic targets are beginning to emerge, and predictive epigenetic biomarkers and next generation epigenome therapeutics are being developed for application in clinical settings for ovarian cancer patients.

Introduction

Epithelial ovarian cancer is the most lethal gynecologic malignancy [1] causing an estimated 15,520 U.S. deaths in 2008 [2]. Most patients are diagnosed with advanced disease, and the five-year survival rate is below 25% for patients diagnosed with stage III - IV epithelial ovarian cancer [1,3]. Most advanced stage patients respond to cytoreductive surgery and platinum-based chemotherapy, however over 70% of women relapse, and platinum-resistant epithelial ovarian cancer is uniformly fatal [4,5].

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Altered epigenetic states are a hallmark of all cancers [6,7], including ovarian [8,9]. Epigenetics is defined as a heritable change in gene expression without alteration of the DNA sequence itself and includes DNA methylation, histone modification, nucleosome repositioning, and most recently posttranscriptional gene regulation by micro-RNAs (miRNAs) [7,10-12]. The transfer of a methyl group to the carbon-5 position of cytosines, almost always within the context of cytosine-guanine (CpG) dinucleotides, is the best-studied epigenetic mark and only known covalent modification of DNA itself in mammalian cells. DNA-associated histone proteins are subject to extensive modifications that mediate the assembly of transcriptionally permissive or repressive (i.e. open or closed) chromatin, and it is now recognized that DNA methylation and histone modifications are intimately linked [7]. The overall epigenetic state (e.g. DNA methylation, histone modification, and miRNA expression) corresponding to a specific cell phenotype is now referred to as the epigenome [13].

Altered epigenetic states in ovarian cancer

The first described epigenetic change in ovarian epithelial cancer was loss of DNA methylation [14]. Global DNA hypomethylation in cancer is largely due to decreased methylation of repeat DNA (non-gene sequences), including centromeric satellite α DNA and juxtacentromeric satellite DNA (classical satellite 2), Alu repeats, and LINE-1 repeats [reviewed in 15]. In ovarian carcinogenesis, the extent of global and satellite DNA hypomethylation was significantly associated with the degree of malignancy [16-18]. Satellite DNA hypomethylation was shown to increase with advanced ovarian tumors and serve as an independent marker of poor prognosis [20]. Hypomethylation may contribute to ovarian carcinogenesis by promoting tumor formation or progression in a number of possible ways, including affecting transposable element activation, DNA/chromosomal rearrangements, tumor suppressor gene or oncogene copy number, and/or altered chromosome conformation [reviewed in 21]. In addition to repetitive elements and DNA satellites, promoter CpG island hypomethylation and gene overexpression has been reported in ovarian cancer. CpG islands are DNA sequences containing an atypically high frequency CpG sites [22]. CpG islands generally lack DNA methylation and are usually but not exclusively associated with gene promoters [6,13,22]. In normal ovarian surface epithelial cells, some CpG islands are methylated and do not express the associated containing genes. Hypomethylation and re-expression of a number of those protein encoding genes in ovarian cancer has been associated with chemoresistance, including MCJ [23], SNCG [24], and BORIS [25]. Hypomethylation of IGF2, an imprinted gene [26], and claudin-4, whose overexpression leads to disrupted tight junctions between epithelial ovarian cancer cells [27], has also been reported in ovarian cancer.

Increased methylation of CpG islands is a common occurrence in epithelial ovarian cancer [8,9], and CpG island hypermethylation is associated with epigenetic silencing during all phases of the cancer process, including tumor initiation, progression and drug resistance [6, 7]. CpG islands aberrantly methylated in ovarian tumors are associated with silencing of genes involved in control of the cell cycle, apoptosis and drug sensitivity, as well as tumor suppressor genes [8]. A number of genes are hypermethylated and down-regulated in ovarian cancer, including the classical tumor suppressors BRCA1 (breast cancer susceptibility gene-1) [28, 29], p16 [30], and MLH1 [29,31], putative/candidate tumor suppressor (RASSF1A, OPCML, SPARC, ANGPTL2, CTGF) [32-36] imprinted genes (ARH1 and PEG3) [37] and proapoptotic genes (LOT1, DAPK, TMS1/ASC, and PAR-4) [38-40], as well as genes associated with cell adhesion (ICAM-1, CDH1 [41,42], cell signaling (HSulf-1) [43,44], genome stability (PALB2) [45], and taxane resistance (TUBB3) [46], and embryonic development (HOXA10, HOXA11 [47].

Methylation microarrays have been used to globally examine DNA methylation in ovarian cancer cell lines and patient samples [48-53]. Those studies have demonstrated that ovarian

tumors contain a large number of hypermethylated loci and that the degree of aberrant methylation (*i.e.*, the total number of methylated genes) is directly correlated with ovarian tumor progression and recurrence. Methylation profiling combined with bioinformatic approaches have also identified specific methylated loci statistically associated with poor progression-free survival in ovarian cancer. As such, “methylation signatures” may contribute to the further classification of ovarian tumors and identification of altered biological pathways for individualizing treatment strategies. With the objective of using methylated loci as biomarkers for monitoring epigenetic therapies [54], we recently developed a model system to examine DNA methylation changes associated with the onset of drug resistance in ovarian cancer [53]. By integrating DNA methylation and gene expression profiles and applying bioinformatic approaches, our pathway analyses suggested significant disruption of tumor-suppressive functions by hypermethylation and upregulation of tumor-promoting cascades by hypomethylation [53]. Such experimental and computational approaches may be highly valuable for identifying key mediators of chemotherapy resistance as potential biomarkers or therapeutic targets.

Altered methylation profiles may be associated with increased or altered activity of the DNA methyltransferases (DNMTs), the family of enzymes that catalyze the transfer of a methyl group to DNA, using S-adenosyl methionine (SAM) as the methyl donor [7]. Although the relationship between gene hypermethylation and altered DNMT RNA levels in ovarian cancer cells is not straightforward [18,55], significant upregulation of DNMTs was observed during acquired cisplatin resistance in ovarian cancer [53], suggesting that aberrant methylation patterns may be associated with increased or altered DNMT activity. Furthermore, targeted downregulation of DNMTs using small interfering RNAs (siRNAs) resulted in loss of CpG hypermethylation and growth inhibition [56], providing functional validation of promoter DNA hypermethylation in ovarian cancer.

BRCA1 is a well-studied gene in both inherited and sporadic ovarian cancer [57]. With regard to tumors, BRCA1 hypermethylation and subsequent gene silencing occurs in 10-15% cases of sporadic disease [58] and correlates with poor clinical outcome [57,58]. However, methylation of BRCA1 and BRCA2 rarely occurs in hereditary ovarian cancer [59], further indicating that promoter methylation is not a frequent “second-hit” in tumors from BRCA1 or BRCA2 carriers [59]. Hypermethylation of BRCA1 has recently been detected in the serum of ovarian cancer patients [32] and serum detection of epigenetic methylation of specific genes (*e.g.*, BRCA1, MLH1 and others) [32,60,61] may serve as predictive markers for patient response to standard therapies, epigenetic therapies [62], or targeted therapies for ovarian cancer [63,64].

DNA methylation and histone modifications regulate numerous normal ovarian functions [65], and altered expression of chromatin-modifying proteins has recently been reported in ovarian cancer cells [66]. Gene silencing by histone modifications, in the absence of DNA methylation, has been reported for GATA4 and GATA6 [67], cyclinB1 [68] and p21WAF1/CIP1 [69]. Similarly, repressive histone modifications (trimethyl-H3K27 and dimethyl-H3K9) together with histone deacetylase (HDAC) enzymes act to down-regulate ADAM19 without CpG island methylation in TGF- β 1-refractory ovarian cancer cells [70], demonstrating that aberrant TGF- β 1 signaling can result in formation of a repressive chromatin environment, without DNA methylation. In addition, genome-wide loss of the repressive trimethyl-H3K27me3 mark was associated with reduced global DNA methylation, allowing platinum re-sensitization of chemoresistant ovarian cancer cells, and allowing for identification of direct target genes of H3K27 methylation-mediated silencing [71]. Complex DNA methylation and histone modification patterns almost certainly contribute to ovarian cancer progression and drug resistance in patients, as loss of H3K27 trimethylation has recently been associated with

poor prognosis in ovarian and other malignancies [72], and gene promoter DNA methylation can be maintained in the absence of this repressive mark [71].

Preclinical epigenetic drugs studies in ovarian cancer

Unlike cancer-associated gene mutations, amplifications, and deletions, DNA methylation and other epigenetic modifications are potentially reversible. Based on extensive findings of aberrant DNA methylation in malignancy, inhibitors of DNA methyltransferases (DNMTs) have been examined as a means of inducing re-expression of tumor suppressor genes and reversal of malignant phenotypes [73]. These drugs are analogues of deoxycytosine possessing various substitutions at their 5-carbons, and effectively prevent transfer of the methyl group. Consequently, upon phosphorylation and incorporation into DNA, DNMTs irreversibly “trap” the methyltransferase enzyme in a transition state complex, which is subsequently eliminated from the cell [73]. Numerous cytosine analogs that covalently and irreversibly bind to the active site of DNMTs are being investigated for their ability to clinically reverse CpG island methylation in cancer and derepress epigenetically silenced genes [54].

Recent preclinical studies have suggested that hypomethylating agents are more effective in combination with conventional chemotherapy, by promoting resensitization of chemoresistant tumor cells. It was hypothesized that this effect is due to re-expression of tumor suppressor genes by promoter demethylation; activation of those genes may then restore drug response apoptotic pathways. Several preclinical models support this hypothesis [74-78]. For instance, in an ovarian cancer model, decitabine and a related epigenetic modulator, zebularine, mediated resensitization of cisplatin-resistant epithelial ovarian cancer cells to platinum [78]. This occurred as a consequence of upregulation of tumor suppressor genes (*RASSF1A*, *hMLH1*) [78]. In another study, treatment with decitabine allowed re-expression of the DNA repair gene *hMLH1* in platinum resistant A2780/CP70 ovarian cancer cells, and xenograft tumors derived from these cells were sensitized by decitabine to cisplatin, carboplatin, temozolomide, and epirubicin [74].

Histone deacetylation is another transcriptional silencing mechanism in ovarian cancer and anticancer effects of HDAC inhibitors (HDACIs) are due to inhibition of deacetylation of nonhistone proteins and subsequent release from epigenetic gene repression [79]. Preclinical studies in ovarian cancer include resensitization of ovarian cancer cells and platinum-resistant xenografts in mice by the HDACIs (belinostat; CuraGen Corp., Branford, CT) [80]; AR-42 (Arno Therapeutics, Parsippany, NJ) [81]), supporting the use of these HDACIs in ovarian cancer clinical trials. Moreover, additive or synergistic effects of HDACI and DNMTI combinations on silenced gene reexpression have been demonstrated [82], suggesting that combining these two classes of epigenetic drugs with conventional therapies may be the most effective approach to use in the clinic [62]. Toward this possibility, one preclinical study showed that a combination of decitabine with belinostat elicited greater platinum resensitization of resistant ovarian cancer xenografts than decitabine alone [83].

Summary

Due to the high levels of recurrence associated with ovarian cancer, there is a need for new treatment options for platinum resistant disease. Therapeutic agents currently under investigation include anti-angiogenesis targeted therapies, antibody-directed therapy, DNA topoisomerase inhibitors, and intraperitoneal administration of chemotherapy [3]. As discussed above, ovarian cancer cells harbor a significantly altered epigenome. Hypermethylation of promoter CpG islands, alterations in histone methylation, and interplay between DNA methylation and histone modifications result in aberrant silencing of tumor suppressor genes in ovarian cancer. Furthermore, these repressive epigenetic alterations are associated with drug resistant disease. Promising pre-clinical results using DNMT and HDAC inhibitors for

chemotherapy resensitization in ovarian cancer cell lines and animal models have been reported, laying the foundation for effective epigenetic drugs in combination with platinum-based agents for overcoming resistance in women with recurrent ovarian cancer.

Clinical experience with hypomethylating agents in ovarian cancer

Based on the success of these inhibitors in the L1210 mouse leukemia model [84], early studies have focused on hematologic malignancies and specifically, leukemias and myelodysplastic syndromes (MDS) [85]. The first DNMTIs studied were 5-azacytidine (5-aza-C, Vidaza) and its deoxyribose analog, 5-aza-2'-deoxycytidine (5-aza-dC, decitabine); both were subsequently approved by the FDA for the treatment of MDS [86-91]. Their effects have been attributed to induction of cellular differentiation, directly related to reversal of epigenetic alterations [92-95]. Early studies with these agents followed the traditional model of a drug studied at or near its maximal tolerated dose (MTD) and utilized high doses of azacitadine and decitabine. Consequently, these trials were limited by toxicity and, particularly, by myelosuppression [96, 97]. However, preclinical models showing that low doses of decitabine or azacitadine induce DNA demethylation, have fueled the redesign of clinical trials using regimens targeting a “*biologically effective*” dose of hypomethylating agents, rather than the MTD. Subsequent trials emulating these *in vitro* findings demonstrated that doses as low as 1/10 of MTD preserved clinical effectiveness, while improving tolerability [98-100].

Hypomethylating agents have been explored in solid tumors both as single agents or in combination regimens [101-102]. In a phase I study in patients with thoracic malignancies, decitabine was administered as a continuous infusion and was dose escalated in a typical phase I schema [103]. The MTD was 60-75mg/m² given over 72 hours, with myelosuppression as the most relevant toxicity. Tumor biopsies obtained before and 24 hours after completion of the infusion demonstrated induction of NY-ESO-1, MAGE-3 and p16 expression in roughly a third of treated patients. That trial provided proof-of-concept that decitabine modulates gene expression in solid tumors, by inducing DNA demethylation. In another phase I study, DNA hypomethylation of a panel of 19 genes was induced by continuous infusion of decitabine, although no single gene showed consistent demethylation among patients [101]. Interestingly, in that study DNA hypomethylation was documented seven days after completion of the decitabine infusion [101], which is consistent with the concept that epigenetic modulation is time-dependent, necessitating progression through several cell cycles. A single study evaluated the activity and tolerability of a demethylating agent in patients with ovarian cancer. Fazarabine (Ara-AC), a nucleoside analogue that consists of the arabinoside ring of 1-beta-D-arabinofuranosylcytosine and the pyrimidine base of 5-azacytidine was administered at a dose of 30 mg/m²/day for 5 consecutive days, on a 28 day cycle to patients with recurrent OC. At this dose the major toxicity was hematologic, with four patients exhibiting grade 4 neutropenia. No complete or partial responses were observed among 19 patients evaluated in this study however 44% of patients had stable disease [104].

To date three clinical trials examined combinations of hypomethylating agents with chemotherapy in patients with ovarian cancer (Table 1). These studies were preceded by a phase I trial demonstrating tolerability of the combination of decitabine and carboplatin in patients with solid tumors [105]. In that trial, decitabine was given as a 6-hour infusion on Day 1 and carboplatin was given as an i.v. bolus on Day 8. DLT was myelosuppression, and the maximum tolerated dose of decitabine was 90mg/m². At that dose, DNA demethylation of a commonly hypermethylated gene (*MAGE1*) was documented in PBMC and in 2 of 6 tumor biopsies obtained before and after treatment [105]. The trial also demonstrated that DNA demethylation was highest between days 8 and 12 after treatment with decitabine, supporting administration of the cytotoxic agent at a later time point. Subsequently, a randomized phase II trial of the UK Cancer Research Group compared the combination decitabine and carboplatin

to single agent carboplatin in patients with ovarian cancer recurring within 6-12 months after first line treatment containing a platinum regimen [106]. Decitabine was given as a 6 hour infusion at 90mg/m² on day 1 and carboplatin was administered at an AUC of 6 on day 8. However due to dose delays caused by neutropenia in patients receiving the combination regimen, the dose of decitabine was de-escalated to 45mg/m². An increased rate of adverse events was noted on the combination arm, with more carboplatin hypersensitivity reactions (64% vs 21%) and more treatment delays for neutropenia (36% vs 10%) compared to patients receiving single agent carboplatin. Less clinical activity was noted in patients receiving the combination regimen (0 objective responses of 11 patients treated by RECIST criteria) compared to patients receiving carboplatin (7 objective responders of 14 patients treated). Biological effects on DNA methylation were not reported.

Concomitantly, a phase I-II trial at Indiana University Simon Cancer Center (IUSCC) investigated the decitabine and carboplatin combination in patients with platinum-resistant or refractory ovarian cancer [107]. To minimize toxicity and enhance the demethylating properties of decitabine, the regimen studied in this trial used low daily doses of decitabine for five days prior to carboplatin. A similar schema of low dose decitabine as single agent had been used for elderly leukemic patients, was well tolerated and induced responses in 54% of treated patients [85]. In that leukemia study there was a gradual and slow time-to-response, consistent with the concept that DNA hypomethylation is time-dependent and requires 2-3 cell cycles to be effective, DNA demethylation being maximal between days 7 and 14. Ten patients were enrolled in the phase I component of the IUSCC trial [107], with nine patients completing at least one cycle of therapy and two dose levels were tested: dose level 1 (10 mg/m²) and 2 (20 mg/m²). DLT consisted of grade 4 neutropenia recorded in 2 of 3 patients treated at dose level 2, therefore dose level 1 was recommended for the phase II component of the trial. The most common toxicities were nausea (80%), allergic reactions (60%), neutropenia (70%), fatigue (50%), anorexia (50%), vomiting (40%), and abdominal pain (40%), the majority being grades 1-2. Grade 3-4 toxicities affecting more than one patient included neutropenia (n=4) and carboplatin allergic reaction (n=3). Efficacy analysis had only an exploratory intent in this part of the study. Patients enrolled on this protocol were heavily pre-treated, with a median number of prior regimens of 5 (range 2-9), had measurable disease and were assessed by RECIST. One complete response (10%) was observed and six patients (60%) had stable disease as their best response. At six months, four (40%) patients were without disease progression. Exploratory biomarker analyses in this study utilized plasma or peripheral blood mononuclear cells (PBMCs) collected at baseline and serially during treatment (on days 5, 8 and 15). Global DNA methylation levels were assessed by MethyLight assay of LINE-1 (long-interspersed) repetitive elements [108] in PBMCs and were reduced in all patients on days 8 and 15, as compared to day 1. Interestingly, no dose effect was observed, suggesting that low dose decitabine (e.g. 10mg/m² daily) was sufficient to induce DNA demethylation, while avoiding excessive toxicity. Additionally, demethylation of five ovarian cancer specific genes (*BRCA1*, *RASSF1A*, *WWOX*, *HOXA10* and *HOXA11*) was examined by using MethyLight in plasma of patients treated on this protocol. Demethylation of *BRCA1* and of *HOXA11* was recorded in plasma collected on days 8 and 15 compared to baseline. The phase II trial examining this combination regimen is ongoing.

Another phase II trial conducted at M.D. Anderson Cancer Center tested a combination regimen consisting of azacitidine given iv at a dose of 75 mg/m²/day for 5 days and carboplatin administered at an AUC of 5 on day 2 on a 28 day cycle [109]. Thirty patients with platinum-resistant or refractory OC were treated on this study. Most prominent side effects were myelosuppression, fatigue and nausea. In this cohort there were 4 objective responses (RR of 14%), of which one was a complete response. The median duration of response was 7.5 months with two patients continuing treatment beyond one year. The long duration of response observed in this study and the proportion of patients without progression recorded in the IUSCC

phase I trial suggest that demethylation by decitabine may play a role in re-sensitizing platinum resistant ovarian tumors to platinum. Future trials testing this concept should incorporate measuring progression free survival as a primary endpoint.

Clinical experience with histone deacetylase inhibitors in ovarian cancer

The dynamic equilibrium between histone acetylation and deacetylation is tightly regulated by histone acetyltransferases (HATs) and histone deacetylase (HDAC). HDAC effects on nucleosomal histones leads to tight coiling of chromatin and silencing of genes implicated in regulation of cell survival and differentiation [110]. Because aberrant HDAC activity has been implicated in cancer, HDACIs are being investigated as anticancer therapeutics [111]. Several HDACIs have been studied, including trichostatin and butyric acid which are active in preclinical models [112], but demonstrated limited clinical activity [113]. Depsipeptide was the first HDACI with demonstrated clinical efficacy [114,115], however this agent was not tested in ovarian cancer. Vorinostat (suberoylanilide hydroxamic acid, SAHA) is a small molecule that binds directly in the HDAC's active site in the presence of zinc. Vorinostat can be administered orally and has excellent bioavailability, major dose-limiting toxicities in phase I trials being anorexia, dehydration, diarrhea, and fatigue [116,117]. Accumulation of acetylated histones post-therapy was demonstrated in PBMCs in patients receiving 200 to 600 mg of oral vorinostat [116,117]. A phase II trial of vorinostat as a single agent in patients with recurrent ovarian cancer relapsing within 12 months after platinum based therapy was performed through the Gynecologic Oncology Group (GOG) [118]. The most significant toxicities included two grade 4 toxicities (leukopenia and neutropenia), 3 constitutional grade 3 toxicities and 3 grade 3 gastrointestinal events. Among 27 women treated on this trial, two survived progression-free for over 6 months and one partial response was recorded. This level of activity was deemed insufficient for patients with recurrent OC and its further investigation as single agent was stopped. Another HDACI that recently entered the clinical arena is belinostat (PDX101), a novel hydroxamic acid HDACI with potent antiproliferative and HDAC inhibitory activities *in vitro* and in xenograft ovarian and colorectal cancer models [119]. In a phase I trial, belinostat was administered i.v. as a 30-min infusion daily on days 1 to 5 of a 21-day cycle to patients with advanced solid tumors, starting with a dose of 150 mg/m² [120]. Dose limiting toxicities were grade 3 fatigue, diarrhea and cardiac arrhythmia at 1200 mg/m², concluding that the maximum tolerated dose was 1000 mg/m² daily for 5 days. Dose-dependent increase in histone H4 acetylation was observed in peripheral blood mononuclear cells, with maximal effects observed at the 900 mg/m² dose, indicating that belinostat is biologically active *in vivo*. Disease stabilization was observed in patients with different malignancies, including sarcoma, renal cancer, thymoma and melanoma, positioning belinostat as an interesting agent for further development in cancer. As many preclinical models suggest that HDACIs exert enhanced anti-cancer activity in combination with demethylating agents [121], chemotherapy [122], or other biological agents [123,124], we believe that further development of HDACIs should include rationally designed combinations.

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Figure 1.

Resensitization of chemoresistant ovarian tumors to carboplatin by epigenetic drug therapy. A. DNA methylation inhibitors (DNMTIs) or histone deacetylase inhibitors (HDACIs), alone or in combination, can reverse platinum resistance in chemoresistant ovarian cancer. Additive or synergistic effects of DNMTI and HDACI combinations on silenced gene reexpression have been demonstrated. B. This chemosensitization is hypothesized to be due to the derepression of tumor suppressor genes (TSG) that were previously silenced by promoter DNA methylation or a transcriptionally repressed (closed) chromatin environment. Epigenetic therapies restore TSG gene expression by creating a more active (open) chromatin environment, reestablishing chemotherapy drug response cascades. See text for details. Black circles, methylated DNA; white circles, unmethylated DNA; green circles, repressive histone modification; stars, activating histone modifications;

TABLE 1
Clinical trials in ovarian cancer testing combinations of demethylating agents and carboplatin

Study	Type	Demethylating Agent	Carboplatin	Toxicity	Efficacy
UK (Ref. 106)	Phase II randomized (n=29) platinum sensitive	Decitabine 90mg/m ² Day 1	AUC 6 Day 8	Neutropenia Platinum allergy	0 responses
IUSCC (Ref. 107)	Phase I (n=10) Platinum resistant	Decitabine 10mg/m ² qd X 5 days	AUC 5 Day 8	Neutropenia Platinum Allergy	1 CR
MD Anderson (Ref. 109)	Phase II (n=30) Platinum resistant	Azacitadine 75mg/m ² qd X 5 days	AUC 5 Day 2	neutropenia Fatigue	1 CR, 3 PR

CR, complete response; PR, partial response