# THE EPIGENETIC REGULATION OF CHEMOTHERAPY RESISTANCE IN MELANOMA

by
**Hussein Tawbi**
B.Sc. in Chemistry, American University of Beirut, 1997
M.D. in Medicine, American University of Beirut, 2001
M.Sc. in Clinical Translational Science, University of Pittsburgh, 2007

Submitted to the Graduate Faculty of the

School of Medicine in partial fulfillment

of the requirements for the degree of

PhD in Clinical Translational Science

University of Pittsburgh

2011

UNIVERSITY OF PITTSBURGH
SCHOOL OF MEDICINE

This dissertation was presented
by

Hussein Tawbi

It was defended on

April 19th, 2011

and approved by

Robert A. Branch, MD, Professor, Department of Medicine

Roger S. Day, ScD, Associate Professor, Department of Biostatistics

Nancy E. Davidson, MD, Professor, Department of Medicine

Mark L. Unruh, MD, MSc, Associate Professor, Department of Medicine

Dissertation Director: John M. Kirkwood, MD, Professor, Department of Medicine

**THE EPIGENETIC REGULATION OF**

**CHEMOTHERAPY RESISTANCE IN MELANOMA**

Hussein Tawbi, MD, PhD

University of Pittsburgh, 2011

Melanoma is rapidly increasing in incidence throughout the world. Early stages are curable with surgical approaches with excellent prognosis. However, a substantial proportion of patients progress to metastatic disease with survival rates of less than 5% making melanoma the culprit for over 65% of all skin-cancer related deaths. Novel agents targeting the immune system and the signaling pathways of melanoma are generating new promise, but chemotherapy remains an important therapeutic alternative, despite low response rates. The resistance of melanoma to chemotherapy is in part due to DNA repair mechanisms that allow cells to survive alkylation damage. Several novel agents targeting the abrogation of DNA repair pathways alone and in combination with cytotoxic agents have been developed with varying measures of success. In this dissertation, we first identified the epigenetic silencing of the DNA mismatch repair (MMR) gene MLH1 as a determinant of response and survival for melanoma patients treated with alkylator-based chemotherapy (dacarbazine/ temozolomide). We then determined the safe dosage of the epigenetic agent decitabine that can be administered in combination with temozolomide. The safety, tolerability and efficacy of the combination of decitabine and temozolomide were evaluated in a Phase II population. We finally determined the pharmacokinetic and pharmacodynamic effects of treatment with the combination of decitabine and temozolomide in the blood and tumor tissues of metastatic melanoma patients.

**TABLE OF CONTENTS**

## LIST OF TABLES

## LIST OF FIGURES

## ACKNOWLEDGMENTS

I have been inspired by my patients, guided by my teachers, intrigued by my colleagues, and supported by my family and friends, for many years to get this work accomplished. I therefore have many people to thank but the following list is a summary:

Dr. John M. Kirkwood who has been a constant source of support, encouragement, and career guidance. He is a true role model; his enthusiasm for science and patient care is simply infectious and I am grateful for having caught it.

Dr. Robert A. Branch who has taught me how to think clearly and has enriched my science in so many ways.

Dr. Roger S. Day who has taught me how to tell truth by numbers and still do it with style; he makes even statistics delightful.

Dr. Nancy E. Davidson for her support at very critical times that allowed for this research to be performed, and her continued support of my training and evolution as a physician-scientist.

Dr. Mark L. Unruh who has been gracious with his time and helped navigate the last few tricky steps before the finish line.

Dr. Merrill J. Egorin who will continue to be remembered and whose legacy I strive to embody. Merrill taught me the audacity to think and then to say what I think.

Dr. Jan H. Beumer, my partner in crime, friend, and most honest critic, without whom I would not have the courage to take many of the steps that formed the body of this work.

Dr. Shama C. Buch, my best friend, collaborator, and sounding board. Shama was there at every step of the way and not only believed in me but also made me believe that I can do it.

To all the patients and their families that in their generosity of spirit, at the most difficult times in their lives, gave me the mandate, the opportunity, and the privilege to continue my efforts towards a cure for cancer.

To my mom, who yelled at me every night when I was working at 2 AM in the morning, and wanted me to work less and enjoy my life more. To be purely and unquestionably loved has been her greatest gift to me.

To my dad, towards whom I always look up, my hero and ultimate fan… I am indebted to you for your wisdom and your compassion, and for the unquenchable energy to achieve what I believe in.

To my brothers and sisters, and their children, which I love so dearly and live with every day of my life. From you I learned that love can transcend space and time.

To my lovely wife, Ursula, my ultimate savior, and my fountain of happiness. You gave me the courage to believe and the energy to go for it. You allowed me to go through it all with a big smile on my face.

To my unborn child, who has been subjected to my science "*in utero*", this is… at the end… my gift to you…

# 1.0 INTRODUCTION

## 1.1 IMPACT OF MELANOMA

Skin cancer has become the most common neoplasm in the United States, with incidence reaching epidemic proportions. The American Cancer Society estimates that more than 2 million new cases of basal cell or squamous cell carcinoma, and more than 68,000 new cases of malignant melanoma are diagnosed annually [1]. An estimated 1 in 5 Americans will develop skin cancer in their lifetime [2]. Overall, the lifetime risk of getting melanoma is about 2% (1 in 50) for whites, 0.1% (1 in 1,000) for blacks, and 0.5% (1 in 200) for Hispanics [3]. With early diagnosis and appropriate management, most skin cancers have an overall 5-year survival rate of 95%. However, melanoma has a significantly higher morbidity and mortality. Although it is the third most common skin cancer, accounting for only 3% of all skin cancers, melanoma accounts for 65% of all skin cancer deaths [4].

Melanoma is the fifth most common cancer in men and the sixth in women in the United States. From 1981 to 2002 the incidence of cutaneous melanoma has increased nearly 2.8-fold. This increase is associated with an increased tendency to perform biopsies on pigmented lesions and has resulted in increased diagnosis of thin (<1 mm) melanoma [5]. It is not entirely clear why we have failed to see a decrease in mortality with earlier detection [6]. The annual incidence of melanoma in the United States between 1998 and 2002 was 17.2 per 100,000 population, a sharp

increase from 5.7 per 100,000 population reported in 1973 [7]. The annual cost of treating newly diagnosed melanoma was estimated to exceed $550 million in 1997 [8]. A more recent analysis estimate the annual cost to be more than $250 million for patients over the age of 65 years alone [9].

## 1.2 ROLE OF CHEMOTHERAPY IN METASTATIC MELANOMA

Early stages of melanoma are surgically curable and adjuvant therapy of high-risk disease is modestly effective in preventing recurrent metastatic disease [10]. Once metastatic, melanoma is incurable with very high disease-fatality rates. The survival rate at 5 years for patients with metastatic disease is <10% with a median survival of 6-9 months [11]. Hydroxyurea was the first agent to get FDA approval in 1967 followed by dacarbazine in 1971, which quickly became the "standard of care" as no other agent, singly or in combination, achieved a survival advantage over single-agent dacarbazine [12].

In this setting, dacarbazine was the only standard option for many patients but for the oncology community in actuality, the standard of care was enrollment on clinical trials. Among the 8 randomized trials in which dacarbazine was used as a comparator arm since 1992, more than 1,000 patients have been treated with dacarbazine with an overall response rate of 13.4% [13]. The oral agent temozolomide (TMZ) is rapidly converted to the same methylating active moiety as dacarbazine (MTIC) and is widely used for the treatment of metastatic melanoma. TMZ has an improved pharmacokinetic and safety profile and good CNS penetration, making it a welcome addition to the armamentarium of the melanoma oncologist and investigative community [14].

Randomized trials have confirmed that TMZ is at least equivalent to dacarbazine but due to its oral mode of administration, this therapy improves modestly on quality of life [15].

Very few agents have shown clinical benefit in the metastatic melanoma setting and none has shown a survival advantage for over 3 decades prior to 2011. Immunotherapy has achieved limited success with high-dose bolus IL-2 leading to durable responses in a small subset of patients (5-15%) [16]. Recent hope was sparked with the benefit observed with the anti-CTLA4 antibody, ipilimumab, in the first-ever metastatic melanoma trial to show improvement of overall survival leading to FDA approval in March 2011 [17]. The approval of ipilimumab will alter the therapeutic landscape of metastatic melanoma and offer effective options for patients with this devastating disease. However, the enthusiasm is tempered by the fact that only 10-15% of patients respond and not all achieve durable responses. Patients that do not respond to ipilimumab or progress on it will still need to be treated with alternative approaches including chemotherapy.

Equally promising has been the advent of highly selective BRAF-targeted agents that have lead to the highest response rates seen in melanoma, in patients that harbor the BRAF mutation [18]. This is highly encouraging although, to date, no durable responses have been described with BRAF inhibitors. Also, those agents are only available for the 40-50% of patients whose tumors demonstrated the mutation. Consequently, patients that a) are wild-type for BRAF; b) have the BRAF mutation but do not respond to BRAF inhibitors; or c) progress on BRAF inhibition, will demand that we have improved options in cytotoxic chemotherapy.

## 1.3 CHEMOTHERAPY RESISTANCE IN METASTATIC MELANOMA

Melanoma resistance to chemotherapy is a major impediment to improving outcomes in patients with this disease. The mechanisms underlying such resistance appear to be closely tied to dysregulation of DNA repair pathways in cancer cells. DNA repair pathways (Figure 1) are present in normal cells to maintain genome integrity. Multiple pathways are activated in response to the genotoxic damage induced by chemotherapy, starting with recognition of DNA damage and culminating in programmed cell death [19]. Failure at any point along this cascade of events can be translated into resistance. A deeper understanding of the DNA repair pathways, implicated as primary mechanisms of resistance, has guided the development of several strategies targeting DNA repair to overcome chemotherapy resistance in melanoma. While inhibition of the direct reversal mechanism, $O^6$-methylguanine DNA-methyltransferase (MGMT) was not met with much success, dual inhibition approaches targeted at MGMT and DNA mismatch repair (MMR) concomitantly may allow the exploitation of its therapeutic potential [20, 21]. The promising results observed in early clinical trials of PARP inhibitors may give this field additional momentum and provide chemotherapy resistance abrogation new and exciting horizons [22, 23].

The successful development of DNA repair inhibitors is still incumbent upon the development of appropriate biomarkers and innovative clinical trial design. Coupling biomarker development with rational combination strategies in carefully designed translational clinical trials offers great hope for improving therapy outcomes in patients with metastatic melanoma.

## 1.4 ROLE OF EPIGENETICS IN CHEMOTHERAPY RESISTANCE ABROGATION

Epigenetics is defined as heritable changes in gene expression without a change in the DNA sequence itself [24]. DNA methylation is an important epigenetic mechanism that has profound roles in gene regulation, development, and carcinogenesis [25, 26]. DNA methylation and histone modifications are involved in the reprogramming of the genome of mammalian cells in cancer [27]. In melanoma at least 50 genes have been identified to date to become silenced by promoter hypermethylation during development and progression of disease [28]. DNA methylation consists of the addition of a methyl group by DNA methyltransferases (DNMTs) to the 5 position of a cytosine ring located in CpG islands [29]. DAC, a potent DNMT inhibitor licensed for myelodysplastic syndromes, reverses promoter methylation and leads to re-expression of epigenetically silenced genes (e.g. MMR proteins MLH1, MSH2, MSH6) with the potential to reverse the chemotherapy-resistant phenotype [30-32].

It can be hypothesized that epigenetically controlled genes can constitute part of an epigenetic marker associated with the chemotherapy-resistant phenotype. It could therefore be expected that agents targeting DNA methylation and histone deacetylation would reverse the resistance phenotype by modulating the epigenetic marker.

## 1.5 STATEMENT OF PROBLEM

A deeper understanding of epigenetic mechanisms underlying chemotherapy resistance in metastatic melanoma provides the opportunity to describe the observed chemotherapy-resistant phenotype as well as to modulate it pharmacologically.

In this dissertation, we will examine: 1) the role of epigenetic silencing of the DNA mismatch repair gene MLH1 in melanoma response to chemotherapy; 2) the safety, tolerability, and efficacy of the addition of the epigenetic agent decitabine to the chemotherapeutic agent temozolomide in metastatic melanoma; 3) the pharmacokinetic and pharmacodynamic effects of the addition of decitabine to temozolomide in patients with metastatic melanoma.

# 2.0 EPIGENETIC SILENCING OF THE DNA MISMATCH REPAIR GENE *MLH1* IS ASSOCIATED WITH SURVIVAL AND RESPONSE TO ALKYLATOR-BASED CHEMOTHERAPY IN METASTATIC MELANOMA

## 2.1 INTRODUCTION

The monofunctional alkylating agents, dacarbazine and temozolomide (TMZ), remain the mainstay of chemotherapy for metastatic melanoma, with response rates of less than 10% [33]. In a recent pooled data analysis of 42 NCI-funded Phase II clinical trials of melanoma, the only parameters correlated with favorable outcomes in metastatic melanoma were clinical characteristics such as performance status, presence or absence of visceral or brain metastases [34]. Those factors are widely considered important for prognostication but do not serve to predict response to therapy.

Both TMZ and dacarbazine (DTIC) are converted after administration to the cytotoxic entity 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MTIC) [35, 36]. MTIC directly methylates DNA resulting in methyl-DNA lesions at specific sites (Figure 1). The $O^6$- methylguanine ($O^6$-meG) is considered to be responsible for most of alkylator cytotoxicity [19, 37]. DNA repair mechanisms can reverse DNA damage and result in cell survival and therefore tumor resistance. $O^6$-meG can be repaired by $O^6$-methylguanine methyltransferase (MGMT) in a process termed direct reversal [38]. $O^6$-meG lesions that are not repaired by MGMT mismatch with thymine and

become a substrate of the DNA mismatch repair system (MMR). A functional MMR is necessary to activate mechanisms culminating in cell death [39-42]. Thus, the efficacy of TMZ or dacarbazine therapy may strongly depend on low MGMT together with high MMR expression [43].

Epigenetic inactivation of genes crucial for control of normal cell growth is a hallmark of cancer cells [44].The transfer of a methyl group to the carbon 5 position of cytosines, almost always in the context of CpG dinucleotides, is the only known epigenetic modification of DNA itself in mammalian cells. Many tumors show increased methylation of CpG islands, CpG rich regions of DNA usually although not exclusively associated with gene promoters, which is associated with epigenetic silencing [45]. CpG islands aberrantly methylated in tumors are associated with silencing of genes involved in control of the cell cycle, apoptosis and drug sensitivity as well as tumor suppressor genes [46, 47]. The epigenetic modulation of genes has been shown to play a significant role in chemotherapy resistance as demonstrated by the marked hypermethylation and silencing of numerous components of DNA repair mechanisms within cancer cells, such as *MGMT*, *MLH1*, *BRCA1*, and *WRN*, in addition to the hypermethylation and silencing of genes associated with apoptosis, such as *RASSF1A*, *DAPK*, *TMS1*, *APAF-1*, and *IGFBP36* [48].

Low MGMT levels have been associated with improved response to TMZ-based therapy [49]. This has been shown to occur through hypermethylation and epigenetic silencing of the CpG island of *MGMT* resulting in improved response of glioma to temozolomide [50]. The MLH1 protein, part of the MMR system, is important in determining sensitivity to a number of important chemotherapeutic agents including alkylating agents and cisplatin [51, 52]. We investigated the role of epigenetic regulation on the expression of DNA repair genes in general,

and *MLH1* in particular, in correlation with alkylator-based chemotherapy outcomes in patients with metastatic melanoma.

## 2.2 PATIENTS AND METHODS

### 2.2.1 Study Design

Using a retrospective cohort study design, we evaluated 66 patients with metastatic melanoma who were treated with alkylator-based chemotherapy at the Melanoma Center of the University of Pittsburgh Cancer Institute between 2000 and 2007. Patients were identified through the institution's medical record data repository. This repository contains whole-text medical records and integrates information from central transcription, laboratory, pharmacy, finance, administrative, and other departmental databases throughout the University of Pittsburgh Medical Center [53]. To meet HIPAA guidelines and ensure patient confidentiality, all data was de-identified (De-ID Software, University of Pittsburgh) using an honest broker system. This study met the criteria for exemption of informed consent by the University of Pittsburgh Institutional Review Board (UPCI 08-009). Tumor samples were obtained from the Health Sciences Tissue Bank (HSTB) at the University of Pittsburgh Cancer Institute. Formalin-fixed paraffin-embedded (FFPE) tissues were available from metastatic lesions on all patients and only patients with available pre-treatment tumor specimens were included in this analysis (n=66).

Chemotherapy regimens studied were primarily single-agent dacarbazine, single-agent temozolomide (TMZ) or dacarbazine-based combinations (including CVD, Cisplatin + Vinblastine + dacarbazine). Patients could have received immunotherapy (interferon or

interleukin-2) in the adjuvant or metastatic setting. Any other form of chemotherapy was excluded from this analysis. Response to chemotherapy was defined as documented objective tumor regression upon treatment with chemotherapy. Patients were considered responders if their objective response by RECIST criteria was partial response or complete response after 2 or more cycles of chemotherapy. Patients with stable disease or disease progression after 2 cycles of chemotherapy were considered non-responders.

### 2.2.2 Illumina Infinium Methylation Analysis

DNA was isolated from melanoma FFPE tissues using the RecoverAll$^{TM}$ Total Nucleic Acid Isolation kit (Ambion, Austin, TX) following the recommended protocol. DNA samples (0.5 μg) were treated with sodium bisulphite using the EZ DNA methylation Gold kit (Zymo), and bisulphite-treated DNA was applied to an Illumina Infinium HumanMethylation27 BeadChip for DNA methylation profiling. This microarray permits the quantitative measurement of DNA methylation for 27,578 CpG dinucleotides spanning 14,495 genes [54].Methylation status of the interrogated CpG sites was determined by calculating β-values, the ratio of the fluorescent signal from the methylated allele to the sum from the fluorescent signals of both methylated and unmethylated alleles.

### 2.2.3 Illumina Whole Genome Gene Expression Analysis

Total RNA was isolated from melanoma FFPE tissues using the RecoverAllTM Total Nucleic Acid Isolation kit (Ambion, Austin, TX). RNA was quantified using Ribogreen RNA quantitation Kit (Molecular Probes, Eugene OR). Whole genome gene expression was carried

out on 250ng of total RNA using Illumina's Whole-Genome DASL Assay which is specifically designed to work with partially degraded RNA as is obtained from FFPE tissue. The Whole-Genome DASL Assay utilizes the whole-genome probe set of Illumina's HumanRef-8 BeadChip which features up-to-date content covering more than 24,000 annotated genes derived from RefSeq (Build 36.2, Release 22).

### 2.2.4 Statistical Analysis

*Whole genome methylation analysis:*

Infinium methylation data was analyzed using Illumina's GenomeStudio software. Background intensity computed from a set of Illumina's internal controls was subtracted from each analytical data point. Unsupervised hierarchical clustering was carried out using GenomeStudio software. Differential methylation was analyzed after applying average normalization.

In an attempt to identify the top methylation sites in combination that can best classify patients, we applied a two-step feature selection procedure and selected the top 10 sites. Leave-one-out cross validation was used to evaluate the performance of the procedure. In each iteration, we leave one patient out to be used as the test set, and used the rest of the patients to select a feature and train a classifier. First, the Wilcoxon rank sum test was applied to the training data. All features with a nominal p-value<0.02 are further considered in the Support Vector Machine (SVM) to select the top 10 feature combination that give the best prediction in the test data set.

*Whole genome gene expression analysis:*

The whole genome gene expression data was initially analyzed using Illumina's GenomeStudio software where background subtraction and quality control checks on the data were performed.

The data was then imported into Biometric Research Branch (BRB) array tools 3.5.0 (http://linus.nci.nih.gov/BRB-ArrayTools.html) for analyses. Class comparison analysis using paired t tests was used to identify genes that are differentially expressed in the responder and non-responder groups. Class prediction algorithms such as Significance Analysis for Microarrays (SAM) and Predictive Analysis for Microarrays (PAM) in BRB array tools were applied to determine whether gene expression patterns could accurately differentiate between responders and non-responders. False discovery rates were calculated using the Korn and Tusher methods [55, 56].

We also used a machine learning approach to select features that could help in classification of the two groups according to the method of Wang et al. [57]. Sequential Minimal Optimization (SMO) algorithm was used with cross-validation for feature selection. Using a training and a validation set, the features which would be selected the maximum number of times when SMO is applied would represent the best features for the entire data. Subsequently, the best features selected are used on the entire data set to test the performance of the predictive signature (cross-validation).

*Analysis of DNA repair genes:*

We used stringent filtering criteria that excluded genes for which less than 20% of expression data have at least a 1.5 -fold change in either direction from gene's median value and/or if more than 50% of the data was missing or filtered out. All other statistical analyses were performed using Stata 11.1 (StataCorp, College Station, TX, USA). The Cox proportional-hazards model was fitted to assess the prognostic and predictive values of the methylation status of the *MGMT* and *MLH1* promoters, as well as MGMT and MLH1 expression. In cases where the proportional

hazards assumption did not hold, non-parametric tests were utilized (Mann-Whitney and Wilcoxon rank sum). Only 6 patients were alive at the time of last follow-up and were censored accordingly.

## 2.3 RESULTS

We identified 66 patients with histologically confirmed metastatic melanoma and for whom tumor tissue obtained prior to initiation of alkylator-based chemotherapy was available. All patients had cutaneous primary melanoma and all were treated with alkylator-based therapy with the majority (68%) receiving single agent TMZ or dacarbazine. The median age was 51 years and there were 45 males and 21 females (Table 1). Patients were classified by chemotherapy response, 18 were considered responders and 48 non-responders.

### 2.3.1 Whole genome methylation analysis

The top 10 methylation sites associated with response were used in a classification algorithm and had an estimated overall accuracy rate of only 60% (Sensitivity =23% and Specificity =74%). Similarly, when the top 10 methylation sites were used to predict survival, the overall accuracy rate was 48% (Sensitivity =39% and Specificity =45%).

### 2.3.2 Whole genome gene expression analysis

Unsupervised hierarchical cluster analysis of whole genome gene expression data did not result in differentiation of responders from non-responders and there were no significant differences between the two groups. Using machine-learning algorithm (SMO), 10 genes were selected as a classifier set: *CDH10, HIST1H3C, FLJ25770, DDIT4L, LRRN3, BHLHB5, EFCBP1, ZNF695, C16orf59, C19orf59*. This 10-gene set had a sensitivity of 83% and a specificity of 94% for identifying the response phenotype with an Area under the ROC curve of 0.917.

### 2.3.3 Analysis of DNA repair genes

The analysis was then restricted to the gene expression and promoter methylation of DNA repair genes involved in the MGMT, MMR and Base Excision Repair pathways, the 3 canonical DNA repair pathways known to be involved in the repair of TMZ-induced DNA damage. Using PAM analysis, only 52 genes passed the filtering criteria and *MLH1* was the only gene that predicted survival at the 0.01 significance level (p=0.0092). Increased *MLH1* expression was associated with a hazard ratio for death of 0.694 (31% decrease in the risk of death). A Cox proportional hazard model was fitted using *MLH1* expression as a continuous variable and identified a high risk and low risk group with highly significant p-value of 0.0099 (adjusted for multiple comparisons using the Bonferroni method).

We subsequently dichotomized *MLH1* expression into 2 groups: high for *MLH1* expression above the mean expression value for the entire cohort and low for *MLH1* expression below the mean. Using the log-rank test, we confirmed that high *MLH1* expression was

significantly associated with improved survival (Figure 2). Although in this dataset, response to chemotherapy was significantly associated with survival (p=0.007). A logistic regression model that adjusted for survival still identified a significant association between high MLH1 expression and objective response to chemotherapy (p=0.045). High MLH1 expression had a sensitivity of 63% and specificity of 81% in predicting survival for an Area under the ROC curve of 74%.

We then investigated the regulation of *MLH1* by promoter methylation. We dichotomized the samples based on median methylation values for the entire cohort. Increased methylation of *MLH1* promoter was independently associated with worse outcomes including decreased survival and resistance to chemotherapy (p= 0.047) (Figure 3). *MLH1* methylation and expression were significantly correlated with lower mean expression in patients with hypermethylated *MLH1* promoter and a higher mean expression in patients with hypomethylated *MLH1* promoter (p=0.001) (Figure 4). The predictive value of both MLH1 gene expression and promoter methylation were assessed independently using ROC curves and have similar sensitivities and specificities indicating that they both reflected the same predictive information.

We also examined the role of *MGMT* promoter methylation and gene expression. Neither of these correlated with response or survival (Figures 5 and 6). Given the sequential nature of the 2 pathways, a model integrating their effects on response and survival was tested and revealed that *MGMT* was not informative and *MLH1* continued to be a powerful predictor of outcome.

*MLH1* promoter methylation was used as part of a CpG Island Methylator Phenotype (CIMP) developed for colorectal and gastric carcinoma utilizing DNA methylation data in five CIMP-specific gene promoters [*CACNA1G, CDKN2A (p16), CRABP1, MLH1, and NEUROG1*] [58]. Since these data were available from our patient population, we derived a CIMP index, which results in values from 0 (indicating no promoter methylation for any gene) to 5 (indicating

promoter methylation at all the genes). The cutoff of ≥4 out of 5 promoters being methylated was used in other cancer types but was not associated with response or survival in our melanoma cohort. However, a CIMP value of 0 (no methylation at the 5 promoters) was in fact associated with better survival ($p<0.0001$).

## 2.4 DISCUSSION

In this study, we have shown that the promoter methylation and gene expression of *MLH1* are important predictors of survival and response to alkylator-based chemotherapy in metastatic melanoma. Gene expression profiling provides a powerful means of tumor classification and has been used to identify previously undetected subtypes of cutaneous melanoma [59], to identify a gene expression pattern that correlates with BRAF mutation status in melanoma cell lines [60], and to characterize the progression of melanoma [61]. More recent studies have examined pretreatment gene expression profiles within cell lines and correlated results with treatment outcome [62-64]. Efforts in the field of melanoma have largely been restricted to candidate genes and not a global genome-wide approach. A recent study by Augustine et al. represents, to date, the most comprehensive approach where the investigators studied a panel of 26 human-derived melanoma cell lines in an effort to evaluate the relationship between temozolomide sensitivity, DNA mismatch repair (MMR) efficiency, and gene expression patterns [65].

The 10-gene signature identified by us in this report performed reasonably well for the prediction of a chemotherapy response phenotype. However, the False Discovery Rate (FDR) for the genes involved ranged from 0.23-0.72, which raises concern about its robustness and requires a larger cohort to confirm its validity.

Hypermethylation of CpG sites, often in promoter regions or CpG islands of tumor-related genes, plays a role in the development and progression of many cancers [50, 66-70]. The detection of hypermethylated genes in tumors has become an important approach to the assessment of candidate tumor-related gene inactivation as well as to disease prognostication and prediction of response to therapy [71-73]. The study by Shen et al. on the correlation of DNA methylation with gene expression analysis using the NCI-60 cancer cell line panel revealed that DNA methylation correlated with gene expression in most cases. This approach illustrates the added advantage of being able to detect genes with low baseline expression as well as genes with multiple alternate transcripts, two situations that are particularly problematic in gene expression profiling. In addition this provides the opportunity to distinguish an epigenetically silenced state from a physiologic transient decrease in gene expression [74].

Several studies have focused on candidate genes such as *MGMT* and *RASSF1A* in cell lines as well as primary tumors [75-78]. A pivotal study by Hegi et al. established the promoter methylation status of *MGMT* as a predictive biomarker for survival in patients with glioblastoma multiforme treated with temozolomide [50]. A study by Mori et al. reported that patients with hypermethylation of the *RASSF1A* and *RAR-ß2* in DNA from the serum of melanoma patients receiving biochemotherapy had significantly worse overall survival than patients with no methylated genes [79].

The role of *MGMT* promoter methylation and/or expression appears to be limited in predicting the outcomes of chemotherapy in metastatic melanoma. Recent reports indicate a role for MGMT in predicting toxicity but not response to chemotherapy [80, 81]. This is not surprising given the fact that the MMR pathway is an obligate pathway to apoptosis after alkylator-induced

DNA damage and its deficiency is likely to be a more relevant mechanism of chemotherapy resistance (Figure 1).

In MMR, the MutSα-complex heterodimer (MSH2, MSH6) binds to the mismatch and recruits an additional heterodimer, the MutLα complex (MLH1, PMS2). As the MMR system does not remove $O^6$-meG, but the nucleotides opposite to it, thymine is inserted again. This results in futile repair attempts and subsequent activation of a signaling cascade, resulting in cell cycle arrest in the G2 phase followed by apoptosis, mitotic catastrophe, or a senescence-like state [82].

MMR deficiency therefore results in clinical drug resistance. Biallelic inactivation of *MLH1* because of a SNP at the acceptor splice site of intron 15 may lead to the disruption of MMR in melanoma [83]. Loss of mismatch repair due to methylation of the *MLH1* gene promoter results in resistance to cisplatin in cell lines in vitro and in human tumor xenografts in vivo [71]. Methylation of the *MLH1* gene promoter is observed in many tumor types [84, 85] and loss of *MLH1* expression is associated with clinical drug resistance in ovarian cancer [86].

In this study, our findings indicate that *MLH1* expression, presumably resulting in a functional MMR, is associated with improved chemotherapy outcomes in metastatic melanoma. We have also identified promoter methylation of *MLH1* to be the most likely mechanism through which *MLH1* is being silenced.

Our results are of particular relevance on several fronts:

1- they suggest a role of the MMR system in melanoma resistance to alkylating agents.

2- they identify epigenetic silencing as the alteration underlying such resistance and therefore offer the opportunity of therapeutic interventions. Specifically, this epigenetic change can be reversed pharmacologically with agents such as decitabine or HDAC inhibitors. In fact, in

an ovarian cancer cell line model, Barvaux et al. showed that cells in which MMR is epigenetically silenced are resistant to TMZ even after MGMT depletion while re-expression with decitabine leads to the reversal of resistance [21].

3- they offer the opportunity of developing a simple and reproducible predictive biomarker of chemotherapy response in melanoma. Specifically, promoter methylation of *MLH1* is a DNA-based test that is more easily reproducible than mRNA expression even in FFPE tumor tissue preserved for years (as in our patient population).

The conclusions of our study are limited by the relatively modest sample size however the results are quite consistent with a well-understood mechanistic model of chemotherapy resistance. Our findings will require validation in an independent cohort, where ideally data can be collected prospectively to avoid biases inherent to retrospective studies.

## 2.5 CONCLUSION

In this study, we have demonstrated the role of the MMR gene *MLH1* as a predictor of survival and response to alkylator-based chemotherapy in metastatic melanoma. A particularly useful application of our strategy is the identification of a subset of patients in whom an epigenetically modifiable molecular alteration (namely promoter methylation of *MLH1*) offers the potential to be transformed into one that is more favorable to chemotherapy thereby abrogating drug resistance. This hypothesis is being actively investigated in patients with metastatic melanoma treated on a prospective Phase I/II clinical trial with the epigenetically active agent, decitabine, in combination with temozolomide at the University of Pittsburgh Cancer Institute (UPCI 07-008).

## 2.6 FIGURES

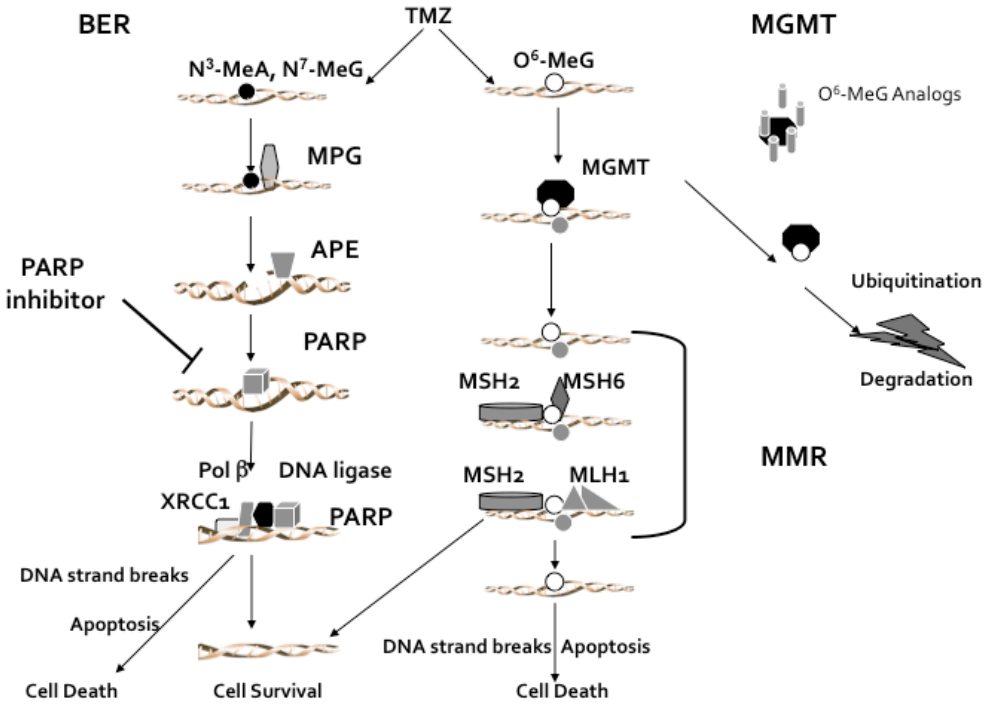


**Figure 1. DNA Repair Pathways**

Three canonical DNA repair pathways are involved in reversing the DNA damage induced by Temozolomide (TMZ). TMZ methylates the DNA backbone at specific residues such as the $O^6$-position of guanine (resulting in $O^6$-methylguanine ($O^6$-meG), $N^7$-methylguanine ($N^7$-meG), and $N^3$-methyladenine ($N^3$-MeA). $N^7$-meG and $N^3$-meA DNA adducts are rapidly and efficiently repaired by the base excision repair (BER) pathway, and normally contribute little to TMZ-induced cell death. MGMT ($O^6$-meG DNA methyltransferase) removes the $O^6$-alkylguanine DNA adduct and restores the guanine to normal. Mismatch Repair (MMR) recognizes the mis-

pair formed during replication of an un-repaired $O^6$-MeG lesion. MMR attempts to repair this mismatch but fails resulting in futile cycles of repair that then initiate cell death.

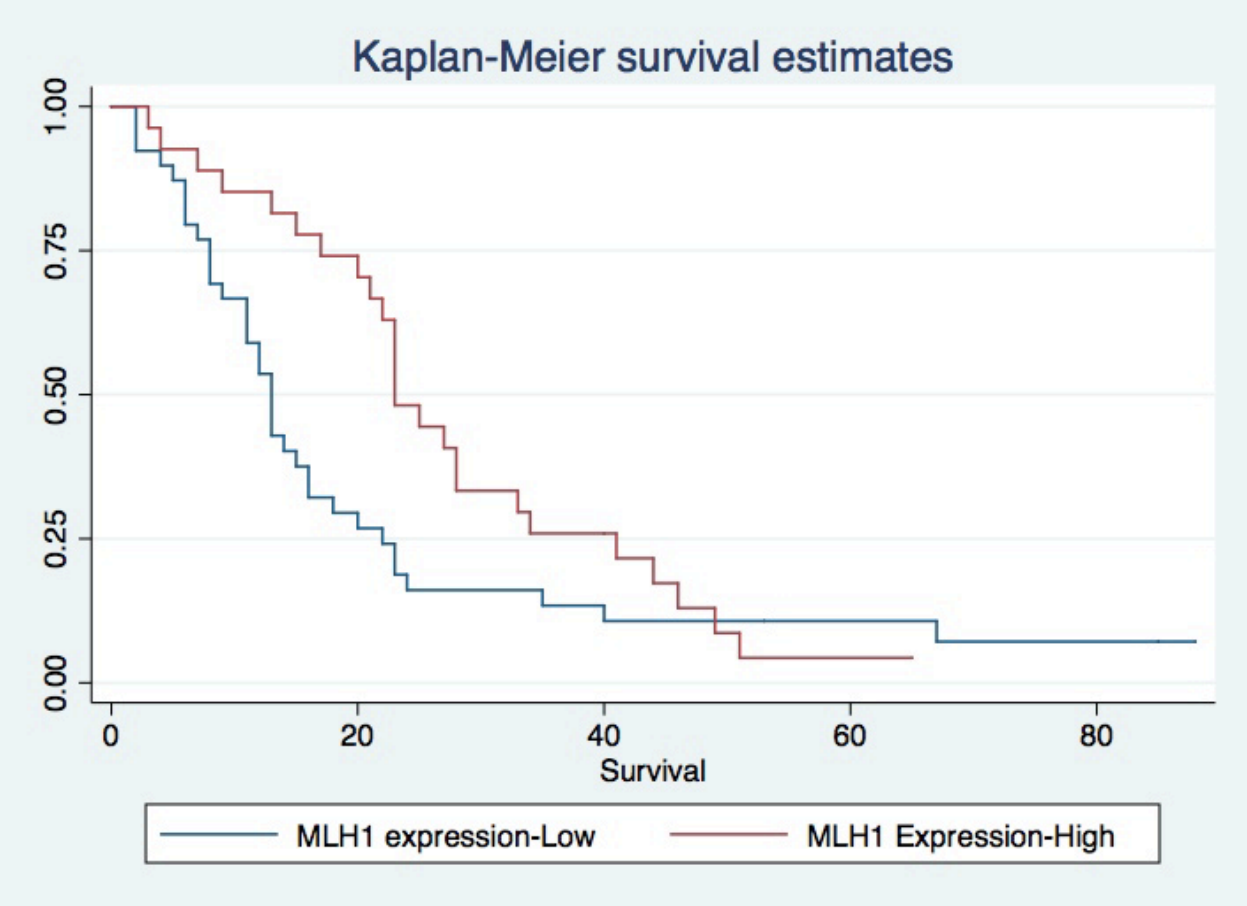


**Figure 2 *MLH1* expression and survival**

Kaplan-Meier Survival Plots with significantly improved survival in patients with high *MLH1* Expression.

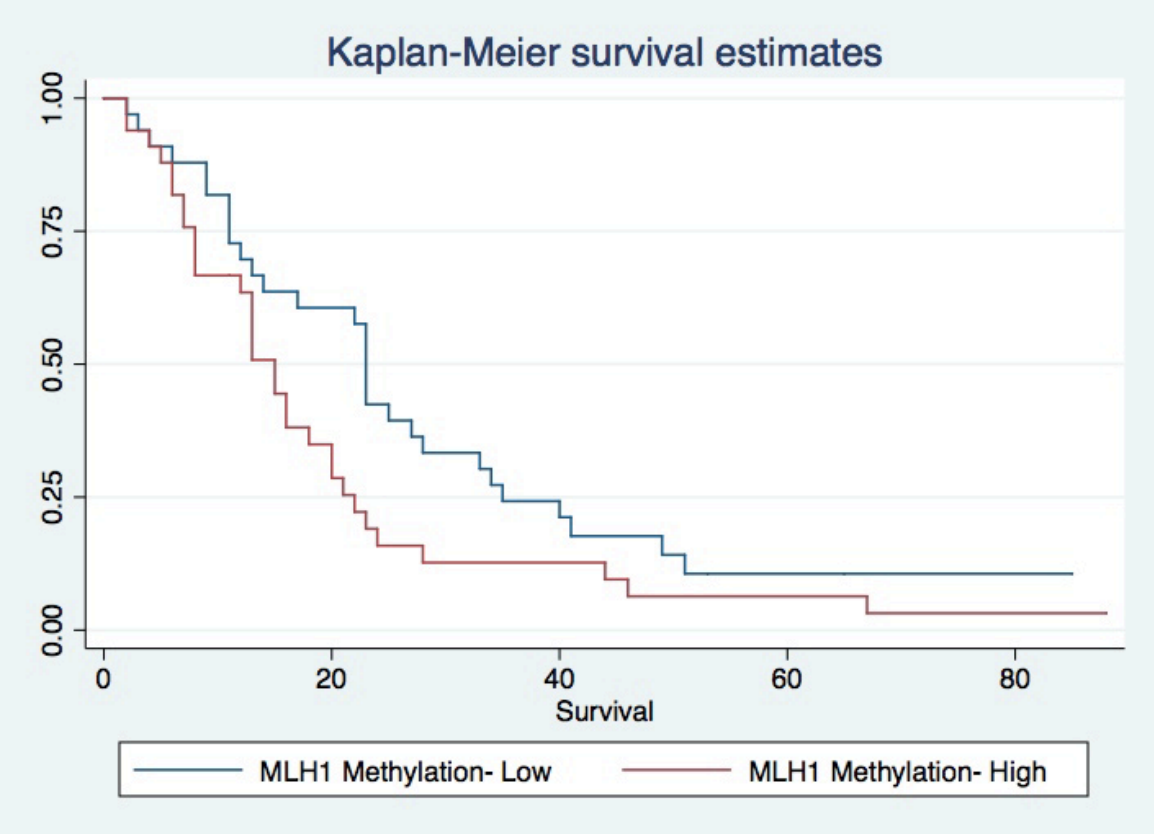


**Figure 3 *MLH1* methylation and survival**

Kaplan-Meier Survival Plots with significantly improved survival in patients with low *MLH1* Promoter Methylation.

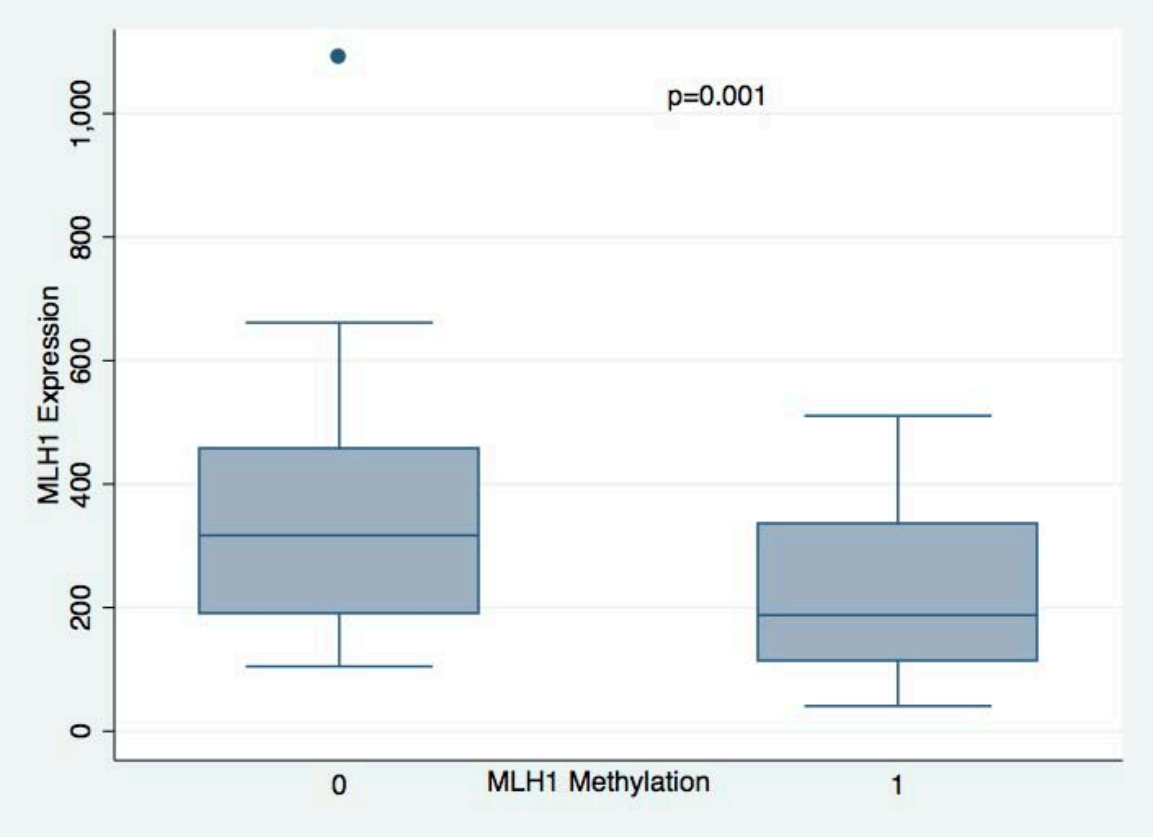


**Figure 4 *MLH1* expression in relation to methylation status of *MLH1***

*MLH1* promoter unmethylated = 0 and methylated =1. Box plot is centered around mean, p-value of 0.001 obtained by one-way ANOVA.

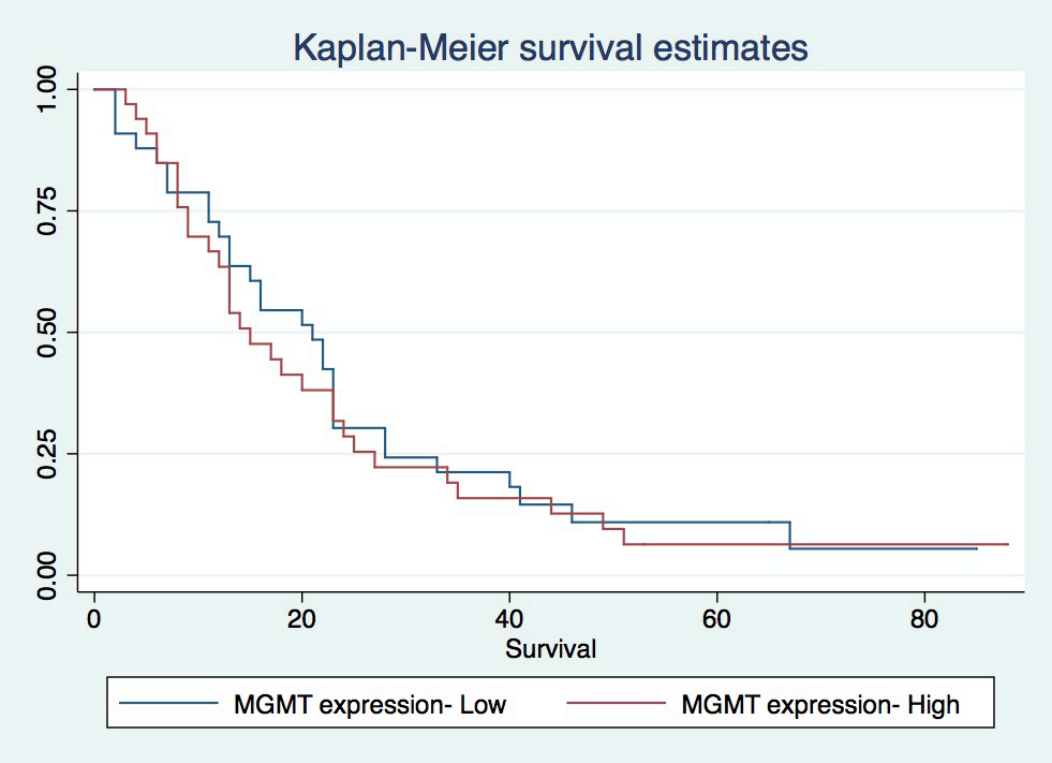


**Figure 5 *MGMT* expression and survival**

Kaplan-Meier Survival Plots showing no difference in survival based on *MGMT* Expression.

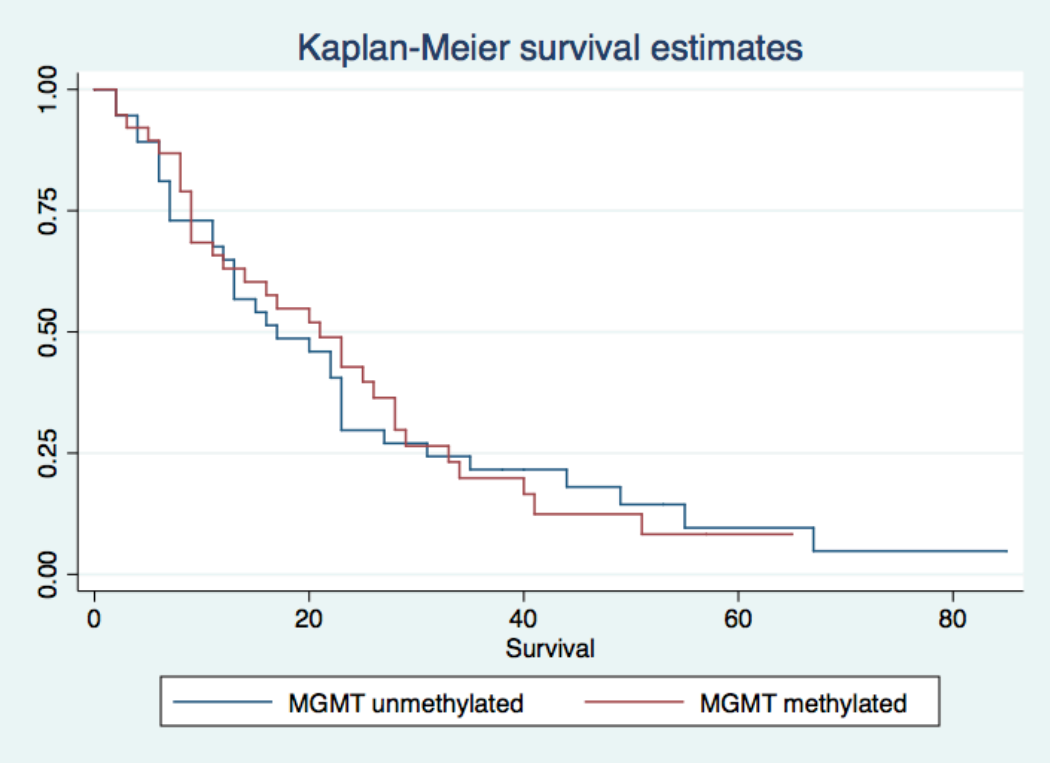


**Figure 6 *MGMT* methylation and survival**

Kaplan-Meier Survival Plots showing no difference in survival based on *MGMT* Promoter Methylation.

## 2.7 TABLES

**Table 1 Characteristics of Study Population**

| | **N** | **Median (Range)** |
|---|---|---|
| Age (years) | 66 | 51 (23-90) |
| | **N** | **(%)** |
| Gender | | |
| Female | 21 | 32 |
| Male | 45 | 68 |
| Response | | |
| Responder | 18 | 27 |
| Non responder | 48 | 73 |
| | | |
| Types of chemotherapy | | |
| Single agent TMZ | 29 | 44 |
| TMZ-based combination | 3 | 5 |
| Single agent dacarbazine | 16 | 24 |
| dacarbazine-based | 18 | 27 |
| | | |
| Survival (months) | | **Median (Range)** |

# 3.0 EPIGENETIC MODULATION OF DNA REPAIR: PHASE I/II STUDY OF DECITABINE (DAC) COMBINED WITH TEMOZOLOMIDE (TMZ) IN METASTATIC MELANOMA

## 3.1 INTRODUCTION

Melanoma incidence is rapidly increasing throughout the world. Based on American Cancer Society estimates, in 2010 there will be approximately 68,130 new cases of invasive melanoma in the United States [87]. The disease will be responsible for over 8,700 deaths in 2010. Prognosis for patients with metastatic disease remains poor, with a 5-year survival rate of 6% and a median survival of approximately 6 months [11]. The first randomized Phase III clinical trial to ever result in improved survival was reported in 2010 with the anti-CTLA4 antibody, ipilimumab, with a median overall survival of 10.9 months while the objective response rate was only 10.9% [17]. Small molecule inhibitors of the mutated BRAF gene have achieved unprecedented response rates approaching 80% and are likely to lead to improved survival as well. However, this approach is limited to patients that harbor the BRAF mutation (40-50% of melanoma patients) and has not resulted in durable responses, with median duration of response that is approximately 6 months [18].

Alkylating agents remain an important therapeutic option for patients with metastatic melanoma that are either wild-type for BRAF or that progress on ipilimumab or BRAF-directed

therapy. Dacarbazine is the only chemotherapeutic agent in current use that is approved by the US FDA for the treatment of metastatic melanoma, with modest response rates not exceeding 7% in large Phase III trials [33, 88, 89]. Temozolomide (TMZ) is spontaneously converted to the same active metabolite 3-methyl-(triazen-1-yl)imidazole-4-carboxamide (MTIC) after oral administration and has shown clinical activity at least equivalent to dacarbazine in melanoma when given in standard schedules [33].

MTIC directly methylates DNA at the $N^7$ and $O^6$ positions of guanine (70% and 6% of base lesions, respectively), and the $N^3$ position of adenine (9%). The $O^6$-methylguanine ($O^6$-meG) base lesion is considered to be primarily responsible for the cytotoxicity of TMZ and is directly reversed by the DNA repair protein, $O^6$-methylguanine-DNA-methyltransferase (MGMT) leading to cell survival and clinical drug resistance [19]. Low-dose, extended-schedule TMZ has been shown to deplete MGMT but was not shown to improve patient outcomes when compared to standard dacarbazine [90].

However, the cytotoxicity of the $O^6$-meG lesion results only when a functional DNA mismatch repair (MMR) pathway exists to recognize the damage and initiate cell death. MMR deficiency is often induced by epigenetic silencing of key MMR genes, such as *MLH1*, through hypermethylation of the promoter region. Promoter hypermethylation can be reversed with hypomethylating agents (such as decitabine (DAC)) leading to re-expression of *MLH1* and sensitization of tumors to TMZ [21]. We hypothesized that the combination of TMZ and DAC will effect dual modulation of DNA repair through the depletion of MGMT and re-expression of MMR proteins. To test this hypothesis, we conducted a Phase I/II clinical trial of the combination of extended-schedule TMZ and DAC to reverse melanoma resistance to TMZ. This is to our knowledge the first attempted dual DNA repair inhibition approach in the clinic.

## 3.2 PATIENTS AND METHODS

### 3.2.1 Study Design

The study was a non-randomized open-label Phase I/II clinical trial conducted at a single institution, the University of Pittsburgh Cancer Institute. The objectives of the Phase I part were: a) to determine the safety, tolerability, and recommended Phase II dose (RP2D) of the combination of extended schedule TMZ and DAC; b) to determine the pharmacokinetics (PK) of the combination of TMZ and DAC; c) to determine, in peripheral blood mononuclear cells (PBMC) and tumor tissue, the pharmacodynamic (PD) effects of the combination of TMZ and DAC on promoter methylation and expression of selected genes. The objectives of the Phase II part were: a) to determine the efficacy, as measured by overall response rate, of the combination of extended schedule TMZ and DAC given at the RP2D to patients with metastatic melanoma; b) to determine the safety profile of the combination of TMZ and DAC at the RP2D; c) to determine the overall survival (OS) and progression-free survival (PFS) of patients with metastatic melanoma treated with TMZ and DAC. The study (UPCI 07-008, NCT00715793 on clinicaltrials.gov) was reviewed and approved by the University of Pittsburgh Institutional Review Board (IRB) and all patients signed informed consent. The study was jointly funded by Eisai, Inc. and Schering Plough Research Institute (now Merck) and supported by the University of Pittsburgh Cancer Institute and the Melanoma and Skin Cancer Program. The investigators designed, conducted, and analyzed the study independently. Toxicity assessments were performed using CTCAE 3.0 and efficacy assessments were according to RECIST 1.0.

### 3.2.2 Patients

Eligible patients had to have non-resectable Stage IIIB or stage IV metastatic melanoma that have not received prior therapy or that have progressed despite prior therapies. Prior biological therapy was allowed, one line of prior chemotherapy was allowed as long as it did not include TMZ or dacarbazine. Patients had to have an ECOG performance status of 0, 1 or 2, and adequate bone marrow, renal, and hepatic function. Patients with treated and stable brain metastases were allowed.

### 3.2.3 Chemotherapy Regimen

Decitabine was administered at the specified dose level, intravenously, daily 5 days a week for the first 2 weeks of a 6-week cycle. TMZ was administered orally at 75 mg/m$^2$ daily for 4 weeks starting on week 2 of a 6-week cycle (Figure 7). The dose of TMZ was constant on both the Phase I and Phase II portions of the study. In the Phase I portion, two doses of DAC were used: 0.075 mg/Kg on dose level 1 (DL1) and 0.15 mg/Kg on dose level 2 (DL2) (Table 2). Dose delays and dose modifications for toxicity were allowed as specified in protocol.

### 3.2.4 Statistical Considerations

The Phase I part of the study sought to determine the maximum tolerable dose (MTD)/ R2PD using a modified version of the standard "up and down" (3+3) dose-finding method using cohorts of 3 patients. At the start of the trial, three patients were to be placed at dose level 1. Only dose-limiting toxicities (DLT) observed in a patient during the first cycle were used for the dose

escalation decisions. Patients were considered evaluable for toxicity if they received 1 complete cycle of therapy, or if they did not complete the cycle secondary to toxicities. Non-evaluable patients were replaced. Only 2 doses levels were to be explored. If DL2 was considered tolerable, dose escalation was to stop and DL2 declared MTD/R2PD.

The Phase II part of the study was designed in 2 stages using a Simon two-stage design. The treatment would be considered worthy of further study if the response rate were 21% or greater. In this circumstance, a risk greater than 15% (β, type II error) for incorrectly determining that the treatment is not worthy of further study is regarded as unacceptable. On the other hand, we would conclude that the treatment is unworthy of study if the response rates were 7% or less; in this circumstance, a risk greater than 10% (α, type I error) for declaring an unpromising treatment to be worthy of further pursuit is regarded as unacceptable. Subject to these constraints, the two-stage design is satisfied by the following criteria:

a) In the first stage, patients will be accrued and treated until there are 14 evaluable patients. The 6 patients accrued at the MTD/R2PD during the phase I part were to be included in the first stage. If no responses are observed, the study will terminate.

b) If 1 or more responses are observed, the study will proceed to a second stage, accruing an additional 20 evaluable patients.

d) If the second stage is completed with a total of 5 or more responses among the 34 evaluable patients, then it will be concluded that the treatment is deserving of further study.

e) Conversely, if the study terminates at the first stage, or if the responses total 4 or fewer, the treatment will not be recommended for further study.

Details of pharmacokinetic and pharmacodynamic sampling and results are reported separately in Section 4.0.

## 3.3 RESULTS

A total of 39 patients were enrolled on the study. The Phase I part of the study enrolled three patients to DL1, however, one patient did not complete cycle 1 secondary to disease progression and was replaced. No DLTs were observed and dose escalation proceeded to DL2. Six patients were enrolled on DL2 and no DLTs were observed. DL2 was therefore declared the MTD/R2PD and enrolment to the Phase II part started. The six patients treated on DL2 were analyzed with the Phase II population. To be evaluable for efficacy patients had to complete 2 full cycles of therapy. Non-evaluable patients were replaced. A total of 35 patients were enrolled on the Phase II part, two were not evaluable for efficacy.

Patient characteristics are summarized in Table 3. The overwhelming majority of patients had metastatic disease and most had poor prognostic features including: M1c disease (31, 88%), brain metastases (42%), male gender (70%), and PS of 1 (70%). The median age was 63.3 years (range 36-78). 51% of patients had received prior immunotherapy whereas only 23% had received prior chemotherapy. The median number of cycles received was 2, 20% of patients received more than 4 cycles; the total number of administered cycles was 101. All patients were off-study at the time of data cutoff on December 31, 2010. Of note, only 2 patients went on to receive ipilimumab subsequently, both of whom had completed 36 weeks of therapy on this trial; and none of the patients received a BRAF inhibitor.

### 3.3.1 Toxicity

Hematologic toxicities were common and represented all grade 3 and 4 toxicities observed (Table 4). Neutropenia was the most common, occurred typically in weeks 4-5 and recovered in

1-2 weeks. Neutropenic fever occurred in only 2 instances, which were managed successfully with antibiotics and growth factor support. Grade 3/4 neutropenia lasted more than 7 days in 6 out of 34 patients evaluable for toxicity on the Phase II part for an acceptable DLT rate of 18%. Temozolomide dose modifications allowed patients to continue being treated on the study, only 2 patients ultimately discontinued therapy secondary to toxicity. Growth factor support was only used in patients with neutropenic fever and otherwise was not allowed. Common non-hematologic toxicities were grade 1/2 fatigue (59%), and grade 1 nausea (54%) (Table 4).

### 3.3.2 Efficacy

Thirty-three patients were evaluable for response. Of those, one patient had a complete response (CR); four had confirmed partial responses (PR), 14 had stable disease (SD) and 14 had progressive disease (PD) (Table 5). The overall response rate (ORR) was 15.2% [90% CI, 6-29%]. The disease control rate (DCR) comprised of CR+PR+SD was 18/33= 58% [90%CI, 39-70%] (Figure 8). The median progression-free survival (PFS) was 2.8 months [95% CI, 2.6-5.6 months], and the 6-months PFS rate was 26% [95% CI, 15-46%] (Figure 10). The median overall survival (OS) has not been reached but was estimated at the data cutoff point to be 15.2 months [95% CI, 11.7-]. The 1-year OS rate was 63% [95% CI, 48-83%] (Figure 9).

## 3.4 DISCUSSION

In this Phase I/II study, DAC was administered safely in combination with TMZ in patients with metastatic melanoma at biologically relevant doses. The combination of DAC and TMZ

achieved improved clinical efficacy in metastatic melanoma patients as compared to single-agent TMZ: the study reached the pre-specified primary endpoint for efficacy based on an objective response rate of 15%, but also modestly improved median PFS and more than doubled the median OS (15.2 months) and the 1-year OS survival rate (63%).

The role of chemotherapy with alkylating agents has evolved in the treatment of metastatic melanoma. TMZ, given in a standard schedule (150-200 mg/m$^2$ daily x 5 days every 28 days), was compared to dacarbazine in a large Phase III trial conducted in the UK by Middelton, et al. [15]. In the intent-to-treat population, median OS was 7.7 months for patients treated with TMZ and 6.4 months for those treated with dacarbazine. Median PFS time was significantly longer in the TMZ-treated group (1.9 months) than in the dacarbazine-treated group (1.5 months) [p=0.012; HR, 1.37; 95% CI, 1.07-1.75]. No major difference in drug safety was observed. TMZ was well tolerated and produced a noncumulative, transient myelosuppression late in the 28-day cycle. The median PFS of 2.8 months observed in our study was modestly improved over that observed with single-agent TMZ both as delivered in the standard schedule (Middleton [33], 1.9 months, 1 sample log rank test p-value <0.0001) and in an extended schedule (EORTC [90], 2.3 months, 1 sample log rank test p-value <0.0001). The improvement we observed in median overall survival (OS) was striking. Specifically, the median OS observed in the Middleton paper for standard schedule TMZ was 7.9 months. The observed median OS in this study (15.2 months) was significantly higher than 7.9 months (1 sample log rank test p-value=0.003). The median OS was also significantly higher than 9.15 months observed in the EORTC trial for extended scheduled TMZ (1 sample log rank test p-value=0.02).

The modulation of DNA repair mechanisms has been the subject of intense investigation given its potential to reverse chemotherapy resistance and improve the efficacy of alkylators.

MGMT was one of the first DNA repair mechanisms that offered an opportunity for modulation. MGMT is a ubiquitously expressed and highly conserved DNA repair protein that is vital in the maintenance of DNA integrity, and is a well recognized mechanism of alkylator resistance. Unlike other DNA repair mechanisms, MGMT does not activate a pathway but is a single protein that recognizes and repairs DNA damage through its specificity for $O^6$- substituted purines [91]. $O^6$-meG analogs were developed with the goal of depleting MGMT by presenting it with decoy base lesions that are themselves devoid of toxicity. However, extensive experience with the intravenous $O^6$-benzyl guanine ($O^6$-beG) in early phase trials confirmed that $O^6$-beG leads to increased myelosuppression that is not paralleled by an increase in efficacy in melanoma, soft tissue sarcoma, multiple myeloma, and glioblastoma multiforme (GBM) [92-102].

$O^6$-(4-bromothenyl)-guanine (lomeguatrib, Patrin®) is a new generation of $O^6$-meG analogs that is orally bioavailable. In a Phase I trial in patients with melanoma conducted at our institution, lomeguatrib was administered with dacarbazine daily for 5 days and escalated to twice daily for 10 days. The MTD of dacarbazine was only 400 mg/m2, <50% of the standard (800-1000 mg/m2) clinical dose [20]. In a recapitulation of the $O^6$-BG experience, no improvement in the efficacy of dacarbazine was observed although a formal phase II trial is yet to be conducted.

A low dose extended-schedule administration of TMZ offered more sustained (although less profound) MGMT inhibition while the total delivered dose of the alkylating agent exceeded the standard clinical doses usually administered over 5-day regimens [103]. This strategy was the basis for a large EORTC randomized Phase III trial in metastatic melanoma where 859 patients were randomized to receive TMZ 150mg/$m^2$/day orally days 1-7 repeated every 14 days ('week on-week off') or dacarbazine 1000mg/$m^2$ IV every 21 days. The preliminary results reported at the

European Society of Medical Oncology (ESMO) Annual Congress revealed a minor increase in response rates (10 vs. 14%), although the extended schedule TMZ did not impart any survival benefit (median OS 9.36 months vs. 9.13 months) [90].

The lack of clinical efficacy observed with the single-pathway inhibition of MGMT could be, in part, due to the dependence of this pathway on a functional MMR system for cytotoxicity to occur [104]. MGMT and MMR have contrasting effects on DNA $O^6$-MeG. The former provides an efficient mechanism of repair. MMR in contrast does not remove the methylated base but transforms the latter into a lethal lesion and activates the apoptotic pathways. If MMR is deficient the $O^6$-MeG lesion can persist without leading to apoptosis and consequently the cell will survive. MMR deficiency occurs primarily through epigenetic silencing of the key MMR genes by promoter methylation. This was shown to be a reversible process through treatment with epigenetic agents such as Decitabine (DAC). DAC is a DNA-methyltransferase-1 (DNMT-1) inhibitor that is approved for the treatment of myelodysplastic syndromes (MDS) and if used at low doses leads to significant DNA hypomethylation [105].

DAC has been reported to induce hypomethylation in tumor xenografts and this was associated with increased sensitivity to carboplatin [71]. A recently reported phase I clinical trial of DAC in combination with carboplatin determined the phase II recommended dose to be DAC IV at 90 mg/m$^2$ (day 1) followed by carboplatin IV at an AUC 6 (day 8) every 28 days. DAC produced a reduction in DNA methylation equivalent to or greater than that observed in the xenograft model [106]. Treatment with DAC in melanoma cells has also been reported to lead to re-expression of products of epigenetically silenced genes such as the MMR protein hMLH1 and therefore lead to a proficient MMR system sensitizing melanoma cells to the cytotoxic effects of chemotherapy [32, 71].

In this Phase I/II study we used a dose and schedule of DAC comparable to a Phase I trial of DAC in combination with high-dose Interleukin-2 that led to significant hypomethylation in patients with metastatic melanoma [107]. Based on this experience, where doses as low as 0.10 mg/kg led to hematologic DLTs, our trial was designed to explore 2 dose levels –one lower (0.075 mg/kg), and one higher (0.15 mg/kg) than this prior dosage. DAC at 0.15 mg/kg was well tolerated and led to the expected incidence of grade 4 neutropenia ~40-50%. This neutropenia lasted more than 7 days in only 18% of patients, and was associated with neutropenic fever in 2 patients. In general patients were asymptomatic and the neutropenia was reversible without the use of growth factors. Full doses of TMZ were administered and dose modifications were infrequent but allowed most patients to complete therapy safely. Only 2 patients discontinued therapy secondary to toxicity, one for neutropenic fever and the other for requiring >2 dose reductions.

The overall survival benefit in this study is unlikely to be due to subsequent therapy, since only ipilimumab is known to improve OS in patients with metastatic melanoma and only 2 of our patients ever received ipilimumab, both after completing 9 months of therapy with TMZ on our study. In order to account for this factor, we examined the 1-year OS rate, which was significantly higher at 63% [95% CI, 48-83%] than the landmark of 25% established by the Korn meta-analysis of prior phase II trials [34].

Patient selection in a non-randomized Phase II trial limits the extrapolation of results such as these; however it should be noted that our patient population exhibited several features that would have resulted in poorer outcomes. For instance, both the UK and EORTC trials excluded patients with CNS metastases while 42% of our Phase II patients had prior evidence of CNS metastases. Also, in the Korn meta-analysis, the presence of visceral disease (M1c) and ECOG

PS>0 were adverse prognostic factors. Visceral disease was present in 88% of our patients and 70% had PS of 1 (Figure 11).

## 3.5 CONCLUSION

We have conducted a Phase I/II clinical trial with DAC in combination with extended-schedule TMZ in patients with metastatic melanoma which is, to our knowledge, the first attempted dual DNA repair inhibition approach to date in the clinic. We have established a recommended Phase II dose of the combination that led to improved clinical outcomes including response rate, clinical benefit rate, median PFS and median OS, and we believe that this data warrants further evaluation in a randomized setting.

## 3.6 FIGURES

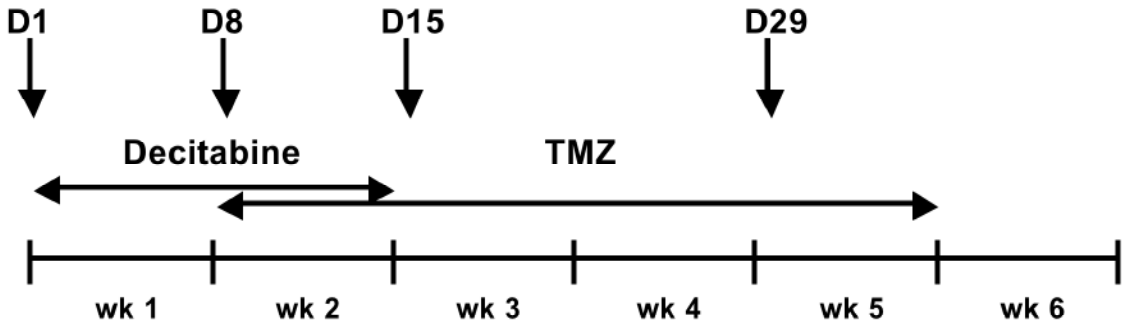


**Figure 7. Treatment Administration Schedule**

Decitabine is given intravenously at the specified dose level 5 days/week for the first 2 weeks; Temozolomide is given orally at 75 mg/m2 daily starting on Day 8 for a total of 4 weeks; week 6 is a rest week. The arrows depict the days on which pharmacokinetic studies were performed (Days 1 and 8) and on which PBMC and optional serial tumor biopsies were collected for pharmacodynamic analyses.

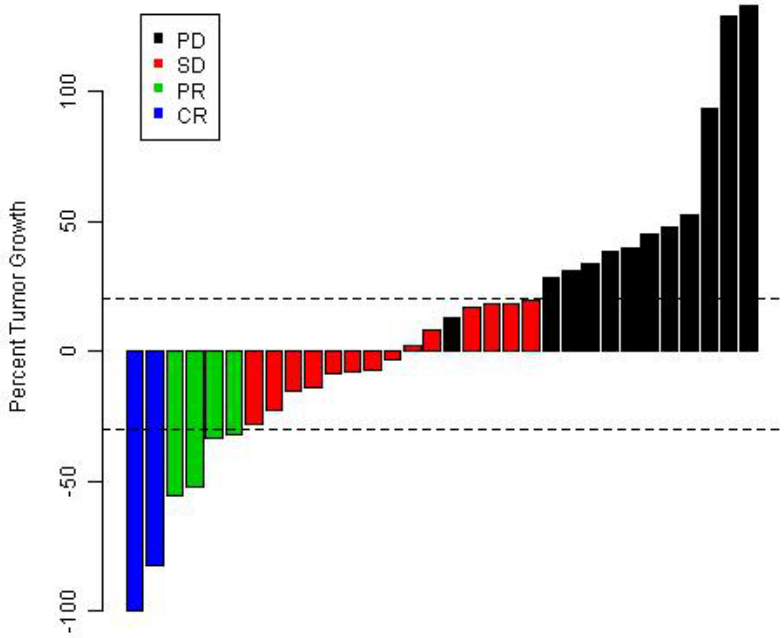


**Figure 8. Waterfall Plot- Best Overall Response**

Best Overall Response by RECIST 1.0 is depicted as percent change on the y-axis. Every bar represents one patient. The horizontal dotted lines represent the RECIST limits for progression (+20%) or objective response (-30%). Complete Response (CR) is represented in blue, Partial Response (PR) in green, Stable Disease (SD) in red, and Progressive Disease (PD) in black. Note that one patient that had SD by RECIST was considered PD (black) as there was evidence of new CNS metastases on evaluation.

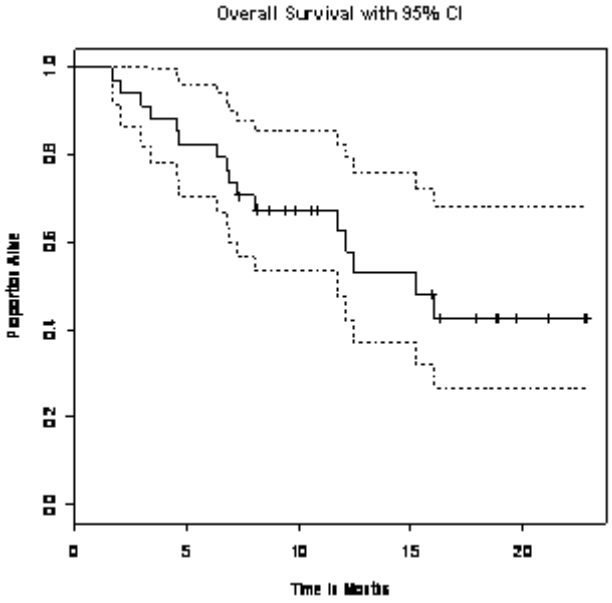


**Figure 9. Kaplan-Meier Curve of Overall Survival with 95% CI.**

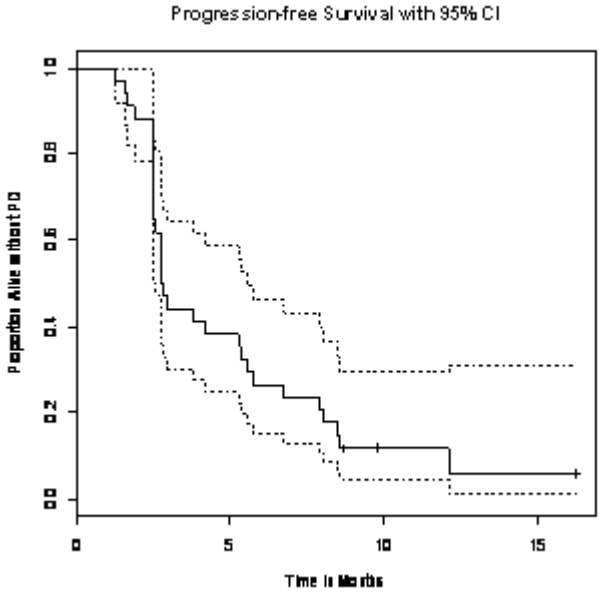


**Figure 10. Kaplan-Meier Curve for Progression-Free Survival with 95% CI**

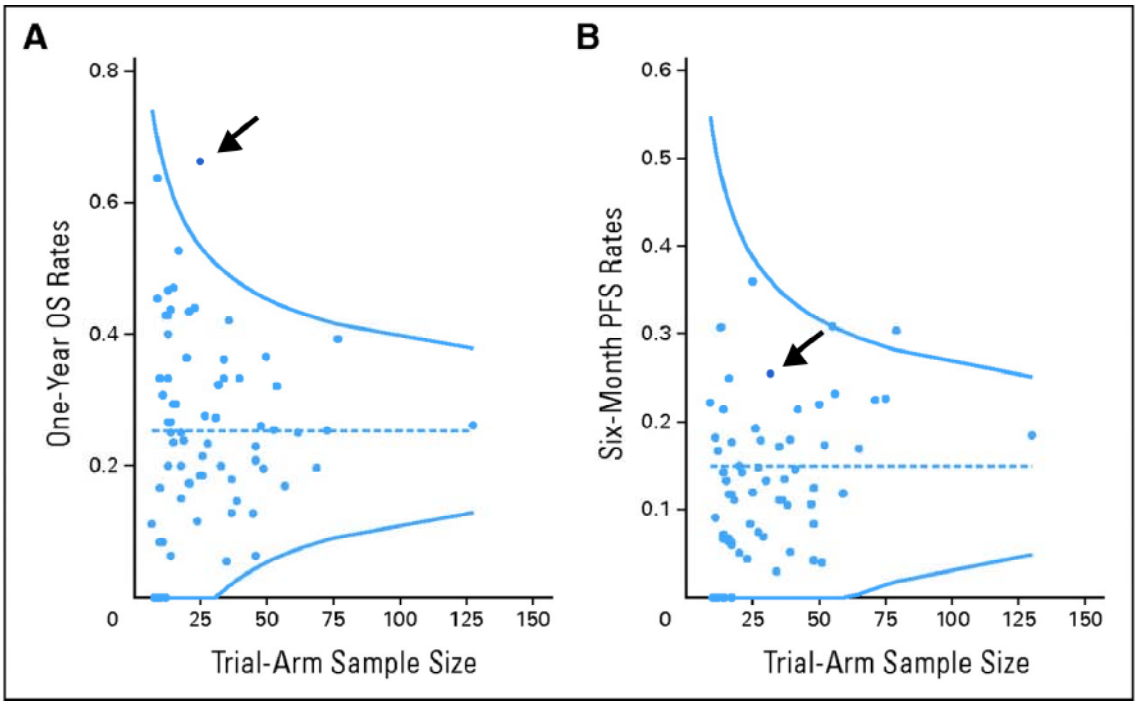


**Figure 11. Current Data in the Context of Korn Model**

Current data presented in context of the meta-analysis by Korn et al., the arrow indicates the data point from the current study.

## 3.7 TABLES

**Table 2. Phase I Dose Levels and Dosing Schedule**

| **Phase I** | **Week** | **1** | **2** | **3** | **4** | **5** | **6** |
|---|---|---|---|---|---|---|---|
| **Level 0** | TMZ PO ($mg/m^2$/day) | - | 75 | 75 | 75 | 75 | - |
| | Decitabine IV (mg/kg qdx5) | 0.0375 | 0.0375 | - | - | - | - |
| **Level 1** | TMZ | - | 75 | 75 | 75 | 75 | - |
| | Decitabine | 0.075 | 0.075 | - | - | - | - |
| **Level 2** | TMZ | - | 75 | 75 | 75 | 75 | - |
| | Decitabine | 0.15 | 0.15 | - | - | - | - |
| **Phase II** | Week | 1 | 2 | 3 | 4 | 5 | 6 |
| | TMZ PO | - | 75 | 75 | 75 | 75 | - |
| | Decitabine IV | 0.15 | 0.15 | | | | |

**Table 3. Patient Baseline Characteristics**

| | | N | Median (Range) |
|---|---|---|---|
| **Age (Years)** | | 39 | 63.3 (36.2-77.4) |
| | | N | (%) |
| **Gender** | Female | 12 | 31 |
| | Male | 27 | 69 |
| **Stage** | Stage IIIC | 2 | 5 |
| | Stage IV-M1a | 4 | 10 |
| | Stage IV-M1c | 33 | 85 |
| **ECOG Performance Status** | 0 | 10 | 26 |
| | 1 | 28 | 71 |
| | 2 | 1 | 3 |
| **Any Prior Treatment** | Chemotherapy | 9 | 23 |
| | Immunotherapy | 20 | 51 |
| **CNS Metastases** | Present | 16 | 42 |
| | Absent | 23 | 58 |

**Table 4. Adverse Events and Number of Patients by Worst Grade (All Cycles)**

| | | Grade | | | | | |
|---|---|---|---|---|---|---|---|
| Category | Type of Adverse Event | 1 | 2 | 3 | 4 | 5 | Total * |
| Blood/Bone Marrow | Hemoglobin | 0 | 4 | 2 | 0 | 0 | 6 |
| | Leukocytes (Total Wbc) | 0 | 8 | 16 | 7 | 0 | 31 |
| | Lymphopenia | 0 | 0 | 1 | 0 | 0 | 1 |
| | Neutrophils/Granulocytes (Anc/Agc) | 0 | 4 | 9 | 19 | 0 | 32 |
| | Platelets | 0 | 3 | 3 | 0 | 0 | 6 |
| Cardiac General | Hypertension | 0 | 1 | 0 | 0 | 0 | 1 |
| | Hypotension | 1 | 0 | 0 | 0 | 0 | 1 |
| Constitutional Symptoms | Fatigue (Asthenia, Lethargy, Malaise) | 10 | 10 | 2 | 0 | 0 | 22 |
| | Fever (In The Absence Of Neutropenia, Where Neutropenia Is Defined As Anc <1.0 X 10e9/L) | 1 | 1 | 0 | 0 | 0 | 2 |
| | Rigors/Chills | 1 | 0 | 0 | 0 | 0 | 1 |
| | Weight Loss | 1 | 1 | 0 | 0 | 0 | 2 |
| Gastrointestinal | Anorexia | 6 | 5 | 0 | 0 | 0 | 11 |
| | Constipation | 13 | 8 | 0 | 0 | 0 | 21 |
| | Dehydration | 1 | 0 | 0 | 0 | 0 | 1 |
| | Diarrhea | 3 | 1 | 0 | 0 | 0 | 4 |
| | Heartburn/Dyspepsia | 3 | 0 | 0 | 0 | 0 | 3 |
| | Ileus, Gi (Functional Obstruction Of Bowel, I.E., Neuroconstipation) | 0 | 1 | 0 | 0 | 0 | 1 |
| | Mucositis/Stomatitis (Clinical Exam), Oral Cavity | 3 | 3 | 0 | 0 | 0 | 6 |
| | Nausea | 22 | 4 | 0 | 0 | 0 | 26 |
| | Taste Alteration (Dysgeusia) | 4 | 0 | 0 | 0 | 0 | 4 |
| | Vomiting | 5 | 2 | 0 | 0 | 0 | 7 |
| Infection/Febrile Neutropenia | Febrile Neutropenia | 0 | 0 | 4 | 0 | 0 | 4 |
| | Infection With Normal Anc Or Grade 1 Or 2 Neutrophils, Skin (Cellulitis) | 0 | 1 | 0 | 0 | 0 | 1 |
| | Infection With Normal Anc Or Grade 1 Or 2 Neutrophils, Upper Airway Nos | 2 | 0 | 0 | 0 | 0 | 2 |
| Neurology | Ataxia (Incoordination) | 0 | 0 | 1 | 0 | 0 | 1 |
| | Confusion | 1 | 0 | 0 | 0 | 0 | 1 |
| | Dizziness | 1 | 1 | 0 | 0 | 0 | 2 |
| Pulmonary/Upper Respiratory | Cough | 6 | 1 | 0 | 0 | 0 | 7 |
| | Dyspnea (Shortness Of Breath) | 4 | 0 | 0 | 0 | 0 | 4 |
| | Hiccoughs (Hiccups, Singultus) | 1 | 0 | 0 | 0 | 0 | 1 |
| Vascular | Thrombosis/Thrombus/Embolism | 0 | 0 | 0 | 1 | 0 | 1 |

**Table 5. Objective Response by RECIST**

| | N | % |
|---|---|---|
| CR: Complete Response | 1 | 3 |
| PR: Partial Response | 4 | 12 |
| SD: Stable Disease | 14 | 42 |
| PD: Progressive Disease | 14 | 42 |
| Overall Response (CR+PR) | 5 | 15% |
| Clinical Benefit Rate (CR+PR+SD) | 19 | 57% |

# 4.0 PHARMACOKINETICS AND PHARMACODYNAMICS OF THE COMBINATION OF DECITABINE (DAC) AND TEMOZOLOMIDE (TMZ) IN PATIENTS WITH METASTATIC MELANOMA

## 4.1 INTRODUCTION

Chemotherapy has limited clinical benefits in the treatment of patients with metastatic melanoma [12]. Chemotherapy resistance is primarily mediated by DNA repair mechanisms [37]. Mounting evidence points to epigenetic silencing of gene expression of key DNA repair genes as mechanisms of resistance. Specifically, down-regulation of mismatch repair (MMR) proteins MLH1, MSH2 and MSH6 has been linked to chemotherapy resistance [71, 108]. Epigenetic silencing through promoter methylation is a dynamic process that is amenable to modulation using agents such as DNA methyltransferase-1 (DNMT-1) inhibitor decitabine (2',5'-deoxy-azacytidine, DAC) [46, 71]. DAC is FDA-approved for the treatment of myelodysplastic syndrome and has been shown to lead to hypomethylation resulting in re-expression of epigenetically silenced genes and associated clinical benefit [109]. We conducted a Phase I/II clinical trial of the combination of extended-schedule TMZ and DAC (UPCI 07-008) to reverse melanoma resistance to TMZ. We hypothesized that the combination of TMZ and DAC will effect dual modulation of DNA repair through the depletion of MGMT and re-expression of MMR proteins, resulting in improved

clinical response. This is to our knowledge the first attempted dual DNA repair inhibition approach in the clinic.

In this translational corollary, we report the results of pharmacokinetic and pharmacodynamic analyses performed on blood and tumor tissues obtained from patients enrolled on UPCI 07-008.

## 4.2 PATIENTS AND METHODS

### 4.2.1 Study Design

The study was a non-randomized open-label Phase I/II clinical trial conducted at a single institution, the University of Pittsburgh Cancer Institute. The objectives of the Phase I part were: a) to determine the safety, tolerability, and recommended Phase II dose (RP2D) of the combination of extended schedule TMZ and DAC; b) to determine the pharmacokinetics (PK) of the combination of TMZ and DAC; c) to determine, in peripheral blood mononuclear cells (PBMC) and tumor tissue, the pharmacodynamic (PD) effects of the combination of TMZ and DAC on promoter methylation and expression of selected genes and correlate these with response. The objectives of the Phase II part were: a) to determine the efficacy, as measured by overall response rate, of the combination of extended schedule TMZ and DAC given at the RP2D to patients with metastatic melanoma; b) to determine the safety profile of the combination of TMZ and DAC at the RP2D; c) to determine the overall survival (OS) and progression-free survival (PFS) of patients with metastatic melanoma treated with TMZ and DAC. The study (UPCI 07-008, NCT00715793 on clinicaltrials.gov) was reviewed and approved

by the University of Pittsburgh Institutional Review Board (IRB) and all patients signed informed consent. The study was jointly funded by Eisai, Inc. and Schering Plough Research Institute (now Merck) and supported by the University of Pittsburgh Cancer Institute and the Melanoma and Skin Cancer Program. The investigators designed, conducted, and analyzed the study independently. Toxicity assessments were performed using CTCAE 3.0 and efficacy assessments were according to RECIST 1.0.

### 4.2.2 Patients

Eligible patients had to have non-resectable Stage IIIB or stage IV metastatic melanoma that have not received prior therapy or that have progressed despite prior therapies. Prior biological therapy was allowed, one line of prior chemotherapy was allowed as long as it did not include TMZ or dacarbazine. Patients had to have an ECOG performance status of 0, 1 or 2, and adequate bone marrow, renal, and hepatic function. Patients with treated and stable brain metastases were allowed.

### 4.2.3 Chemotherapy Regimen

Decitabine was administered at the specified dose level, intravenously, daily 5 days a week for the first 2 weeks of a 6-week cycle. TMZ was administered orally at 75 mg/m$^2$ daily for 4 weeks starting on week 2 of a 6-week cycle (Figure 7). The dose of TMZ was constant on both the Phase I and Phase II portions of the study. In the Phase I portion, two doses of DAC were used: 0.075 mg/Kg on dose level 1 (DL1) and 0.15 mg/Kg on dose level 2 (DL2) (Table 2).

### 4.2.4 Pharmacokinetic Sampling

Samples for pharmacokinetic analyses were performed on the first 14 patients. On days 1 and 8, blood samples (5 ml) were collected using pre-chilled heparinized tubes pre-loaded with tetrahydrouridine (THU) (50 uL of 10 mg/mL in saline) at 0, 15, and 30 minutes (just before the end of infusion); and 5, 15, 30, 45, 60 minutes, 2, 4, 6, and 23 h after the end of infusion (the last sample is taken immediately before Day 2 decitabine administration). Blood samples were centrifuged immediately at 4 °C. Following centrifugation, on day 8 only, a 1-ml volume of plasma was transferred into a plastic tube containing 0.03 ml of 8.5% phosphoric acid followed by brief vortexing (for temozolomide quantitation). The rest of the plasma (all on day 1) was aspirated and transferred to a tube for decitabine quantitation. Samples were frozen immediately and stored at –80 °C until analysis.

Temozolomide concentrations were determined according to the assay described by Kim et al. [110] with the following modifications. A 100 µL aliquot of the sample was placed into a 1.5-ml microcentrifuge tube. To this, 10 µL of 1 N hydrochloric acid, and 10 µL of 1 µg/mL ethazolastone internal standard solution in water were added. After addition of 1 mL of ethyl acetate, the sample was vortexed for 10 minutes, followed by centrifugation at 4500 x g for 5 min at room temperature. The supernatant was transferred to 12 x 75 mm glass culture tubes and evaporated to dryness under a gentle stream of nitrogen at 37 °C. The dry residue was taken up in 100 µL mobile phase by sonication for 2 min. The sample was transferred to an autosampler vial before injection of a 20 µL aliquot. This assay was linear, accurate (94-107%), and precise (coefficient of variation 0.7-3.6%) in the range of 0.1 to 20 µg/mL. Quality control samples were employed at 0.2, 3, and 15 µg/mL.

Decitabine concentrations were determined by XenoBiotic Laboratories, Inc. using a validated method which utilizes a solid phase extraction (MCX) procedure to extract the analyte from 200 µL of human plasma, a reverse phase HPLC column to separate Decitabine and IS (5-Azacytidine) from the matrix, and an LC MS/MS instrument operating with positive ESI-MRM mode to quantify Decitabine. The calibration curve ranges from 1 ng/mL to 100 ng/mL for Decitabine. Decitabine plasma pharmacokinetic parameters were calculated from the data by non-compartmental methods with PK Solutions 2.0™ (Summit Research Services, Montrose, CO, USA).

Temozolomide plasma pharmacokinetic parameters were calculated from the data by compartmental methods. An MLEM population pharmacokinetic 1-compartment, open, linear model with 1st order absorption was fit to the temozolomide concentration versus time data with the ADAPT 5 software for pharmacokinetic/pharmacodynamic systems analysis [111]. The maximum likelihood option in ADAPT 5 was used for all estimations, and parameters were assumed to be log-normally distributed. Based on the schedule of chemotherapy administration patients were not expected to receive temozolomide as a single agent and pharmacokinetic sampling for temozolomide was in the presence of decitabine. To account for possible interactions, we used a population pharmacokinetic analysis approach in which temozolomide exposure was predicted using subject characteristics according to Ostermann et al. [112], based on sex and body surface area (BSA). Predicted temozolomide exposure was then compared to observed exposure to determine whether decitabine affected temozolomide exposure.

For decitabine, only the data from patients with full pharmacokinetic profiles on both days were used in subsequent statistical analyses comparing the two days (Day 1 decitabine alone; Day 8 decitabine in the presence of temozolomide). For temozolomide, apparent

clearance, distribution volume and half-life data from Day 8 (after 7 days and in the presence of decitabine) were compared with those values predicted based on patients demographics. Statistical analyses for pharmacokinetic parameters and concentration values were performed using SPSS 17.0 for Windows (SPSS Inc., Chicago, IL). Values were compared with a two-tailed, paired exact Wilcoxon signed rank test, where a $P < 0.05$ was considered significant.

### 4.2.5 Pharmacodynamic Sampling

Pharmacodynamic sampling was performed on all accrued patients. During cycle 1 on days 1, 8, 15, and 29, whole blood was collected and processed to isolate peripheral blood mononuclear cells (PBMC). Pre-dose 10-ml blood samples were collected in Vacutainer CPT™ cell preparation tubes. PBMCs were isolated by centrifugation at 1500 x g for 25 minutes at room temperature. Isolated PBMCs were washed twice with phosphate-buffered saline at $4^{o}$ C, and stored at –80 ºC before further processing. Core biopsies of tumor samples were obtained from consenting patients with accessible, evaluable disease, on days 1, 8, 15, and 29 of the first cycle. Biopsies were optional in Phase I and Phase II for all consenting subjects.

*Illumina Infinium Methylation Analysis*

DNA was isolated from melanoma tissues using ArchivePure DNA Cell/Tissue kit (5Prime Inc., MD, USA). DNA samples (0.5 μg) were treated with sodium bisulphite using the EZ DNA methylation Gold kit (Zymo), and bisulphite-treated DNA was applied to an Illumina Infinium HumanMethylation27 BeadChip for DNA methylation profiling. This microarray permits the quantitative measurement of DNA methylation for 27,578 CpG dinucleotides spanning 14,495 genes[9]. Methylation status of the interrogated CpG sites was determined by

comparing β-values, the ratio of the fluorescent signal from the methylated allele to the sum from the fluorescent signals of both methylated and unmethylated alleles.

*Illumina Whole Genome Gene Expression Analysis*

Total RNA was isolated from melanoma tissues using the PerfectPure RNA Tissue kit (5Prime Inc., MD, USA). RNA was quantified using Ribogreen RNA quantitation Kit (Molecular Probes, Eugene OR). RNA quality was also evaluated by RNA Integrity Number using the Agilent Bioanalyzer. Whole genome gene expression analysis was carried out using the Illumina HT-12 Expression BeadChip which targets more than 25,000 annotated genes with more than 48,000 probes derived from the RefSeq (Build 36.2, Rel 22) and UniGene databases.

*Whole genome methylation statistical analysis:*

The BeadChip images were scanned by Illumina's BeadStation system and the data were extracted into GenomeStudio software. Background normalization was conducted using the negative control signals from each well. The level of methylation (β) is a measure of the ratio (represented as a value between 0 and 1) of methylated-probe signal to total locus signal intensity. CpG loci with a detection p value of $p<0.01$ were not included in the analysis as a quality control measure. The difference in average β for each locus was calculated for every sample for pre and post treatment comparisons. A change in average β of 0.2 in either direction was considered a measureable outcome in this preliminary analysis according to quality control measures recommended by Illumina.

*Whole genome gene expression statistical analysis:*

Image processing and raw data extraction were performed using the Illumina GenomeStudio software. The data were normalized for background correction, plate scaling and chip-to-chip variation. The number of genes detected in each sample (probe signal significantly

greater than average signal from negative controls with $p<0.05$), was used as a measure of the quality of the results. Genes with a detection p value of <0.05 were not included in the analysis. Fold differences were calculated for all included genes for every sample. A 1.5 fold difference cut off in either direction was applied to each pre and post treatment comparison.

## 4.3 RESULTS

Pharmacokinetic data was available for decitabine in 15 patients and for temozolomide in 14 patients, see Tables 6 and 7. For decitabine analysis, less than 8% of $AUC_{0\text{-inf}}$ was extrapolated.

We did not observe statistically significant changes in the decitabine pharmacokinetic parameters between day 1 and day 8 (Figures 12 and 13). Within-subject variability for decitabine $C_{max}$ and clearance was 31% and 31%, respectively. Likewise, we did not observe statistically significant differences between observed apparent clearance and predicted apparent clearance for temozolomide (Figure 14). We did observe significant differences between observed and predicted temozolomide volume of distribution and half-life. Average pharmacokinetic profiles are displayed in Figure 15.

In 6 patients, tumor tissues were available from pre- and post-treatment. Table 8 summarizes the time points where tumor and matched PBMCs were obtained. Analysis of global DNA methylation revealed that treatment with DAC led to changes in DNA methylation in both directions, with some CpG sites becoming hypermethylated and others hypomethylated (Table 9). Hypomethylation was more prominent and ranged from 2.4% to 10.6% of all CpG sites while hypermethylation ranged from 0.7% to 6.5%. Net hypomethylation occurred in 11 out of 13 instances (85%) and was most prominent at Day 29 although it was present as early as Day 8.

Paired PBMC samples showed an overall net hypomethylation although this was much less prominent being mostly in <1% of all CpG sites and did not coincide with the degree of hypomethylation observed in tumors. Since there were only 6 patients, it was difficult to identify a pattern that correlated with clinical response.

Global gene expression in tumor tissues was affected by treatment with DAC with genes being differentially expressed in either direction (Table 10). Surprisingly, the direction of most samples was rather towards decreased expression in most cases with decreased expression ranging from 0.2% up to 20.9% of all genes while increased expression ranged from 0.04% to not more than 2%. Interestingly, the patients that showed a net increase in expression both had experienced dose-limiting toxicities during cycle 1.

We investigated the changes in promoter methylation and gene expression of all DNA repair genes with special emphasis on MGMT and MMR genes but there was no clear pattern in the changes observed (data not shown).

We then examined the changes in promoter methylation and gene expression of p16 and Hemoglobin F, both implicated in the response to decitabine and clinical benefit in patients with MDS, AML and sickle cell disease [113-115]. Treatment with DAC lead to hypomethylation of p16 and HgF promoter regions in 5 of 6 cases (83%) and this was equally associated with increased gene expression as high as 8 fold in the case of HgF.

## 4.4 DISCUSSION

In this study, we have shown that DAC can be administered safely in combination with TMZ in patients with metastatic melanoma at biologically relevant doses. We have demonstrated that the

2 agents do not affect each other's exposure using extensive PK sampling and population PK analysis. We also observed the pharmacodynamic effects of DAC in tumor tissues at the dose level that was associated with improved clinical efficacy.

Chemotherapy with alkylating agents continues to have a role in the treatment of metastatic melanoma. In this Phase I/II study we used a dose and schedule of DAC comparable to a Phase I trial of DAC in combination with high-dose Interleukin-2 that led to significant hypomethylation in patients with metastatic melanoma. Based on this experience where doses as low as 0.10 mg/kg lead to hematologic DLTs, our trial was designed to explore only 2 dose levels one lower (0.075 mg/kg) and one higher (0.15 mg/kg). DAC at 0.15 mg/kg was well tolerated and led to the expected incidence of grade 4 neutropenia of around 40-50%.

The combination of DAC and TMZ achieved improved efficacy and the trial reached the pre-specified primary endpoint for efficacy based on an objective response rate of 15%. The observed median OS in this study was 15.2 months and was significantly higher than 7.9 months (1 sample log rank test p-value=0.003). We have investigated the underlying mechanisms for this improved therapeutic benefit that the addition of DAC brought.

The exposures observed in this study for temozolomide clearly agree with the exposures reported in the literature [112]. The observed volumes of distribution did significantly differ from those predicted, and consequently the same applied to the half-life, which was calculated from clearance and volume of distribution. However, the volume of distribution does not affect the total exposure and the observed statistical difference is therefore not considered relevant.

The clearance and distribution volumes for decitabine observed in this study agree closely with literature values [116]. The different half-lives between dose level 1 and 2 are attributable to the longer time that plasma concentrations could be followed in patients on dose

level 2, resulting in capture of a more true terminal phase. Based on the PK analysis, there is no indication that DAC increased exposure to TMZ and therefore this benefit cannot be ascribed to higher alkylator effect.

Low doses of DAC did hypomethylate melanoma tissues at both dose levels although only one paired sample was available from dose level 1. This level of hypomethylation was not as profound as is seen with hematologic malignancies which is likely due to the smaller growth fraction of solid tumors as DAC requires cell division to be incorporated into the DNA and inhibit DNMT1. This was manifested in our study by the extent of hypomethylation since more profound hypomethylation at Day 29 as compared to Day 8. It is not clear why this did not result in significant overall increase in gene expression. However, the well documented effect of DAC on specific genes such as p16 and HgF, was reproduced in our data suggesting that DAC did achieve pharmacodynamic effects in melanoma tissue at the RP2D.

Our hypothesis that the improvement in TMZ efficacy is mediated by the effect on DNA repair genes was not substantiated by the available data with the caveat that we have a very limited sample size and our analyses were primarily descriptive.

## 4.5 CONCLUSION

We have conducted a Phase I/II clinical trial with DAC in combination with extended-schedule TMZ in patients with metastatic melanoma. We have established a recommended Phase II dose of the combination that led to improved clinical outcomes including response rate, clinical benefit rate, median PFS and median OS. This improvement in clinical outcome was not

secondary to increased exposure of either drug and was not clearly related to changes in the methylation pattern or expression of DNA repair genes that were studied.

## 4.6 FIGURES

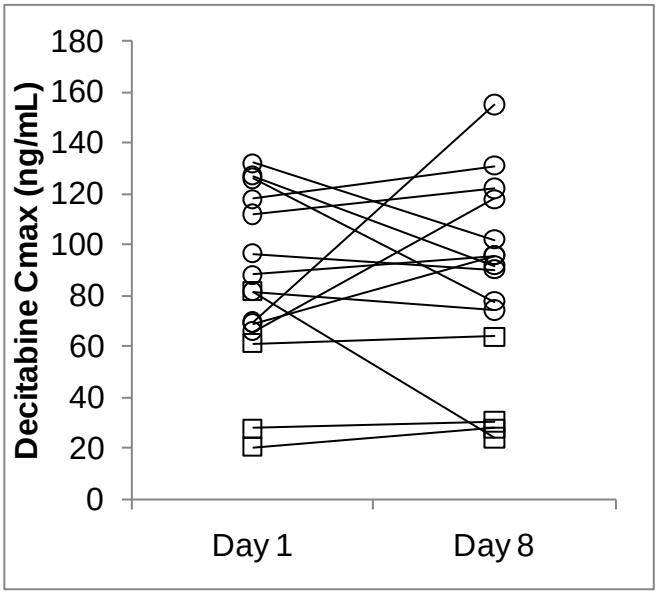


**Figure 12. Decitabine Concentrations (Cmax) on Days 1 and 8**

Intra-individual changes of decitabine $C_{max}$ between Day 1 (decitabine alone), and Day 8 (decitabine with temozolomide); dose level 1 (□) and dose level 2 (○); $P = 0.847$.

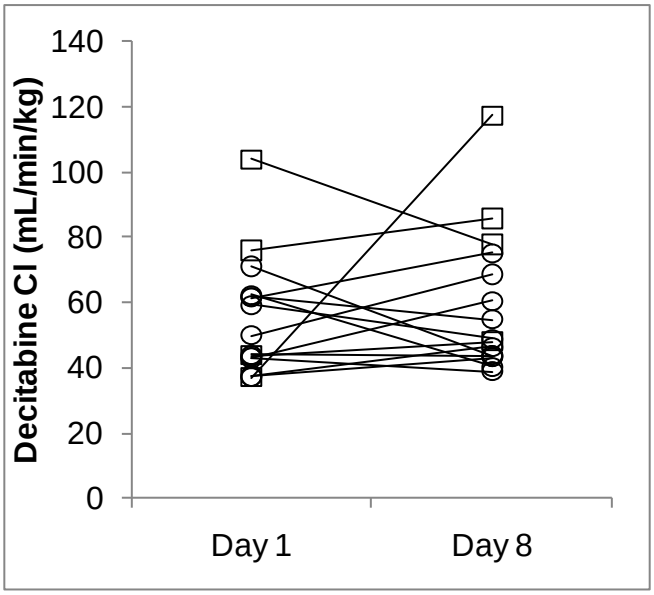


**Figure 13. Decitabine Clearance on Days 1 and 8**

Intra-individual changes of decitabine clearance between Day 1 (decitabine alone), and Day 8 (decitabine with temozolomide); dose level 1 (□) and dose level 2 (○); $P = 0.639$.

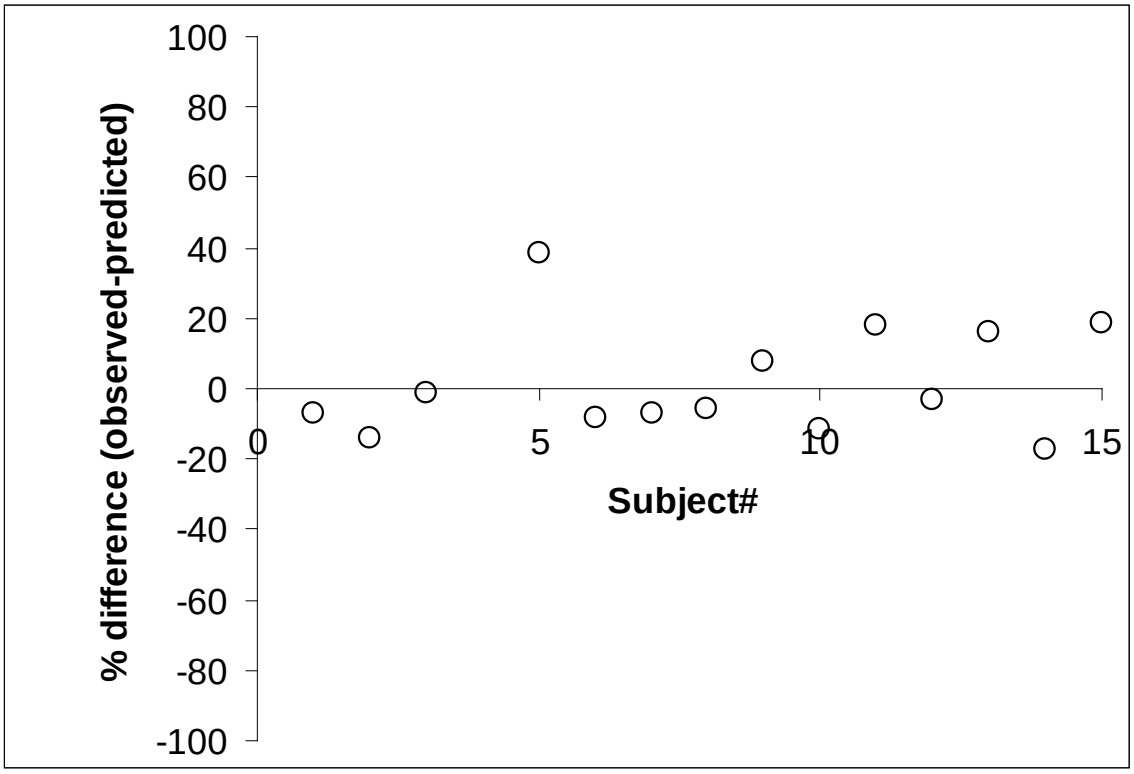


**Figure 14. Temozolomide Population Pharmacokinetics Analysis**

Difference between predicted and observed temozolomide plasma exposure ($AUC_{0\text{-}inf}$). The average difference was 1.6%; $P = 0.855$.

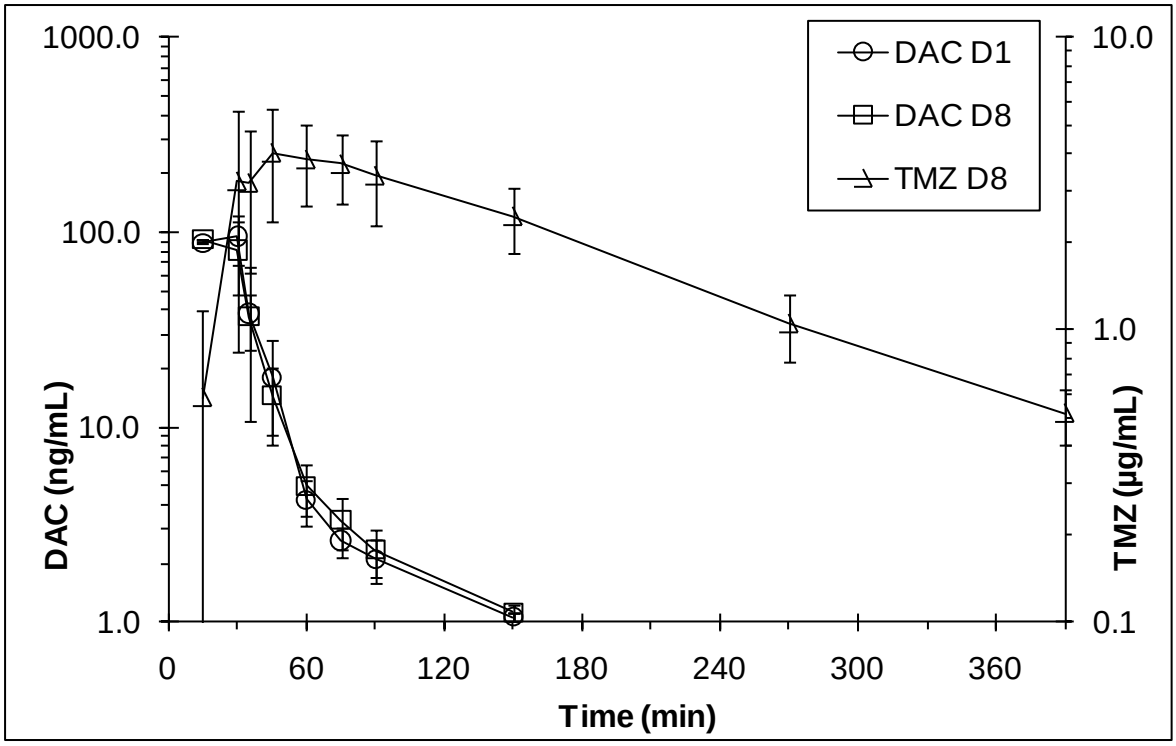


**Figure 15. Concentration vs Time Profiles for Decitabine and Temozolomide**

Concentration versus time profile based on average (±SD) concentration data of 14 patients receiving 200 mg/m$^2$ temozolomide (TMZ) PO with decitabine (DAC).

## 4.7 TABLES

**Table 6. Decitabine Pharmacokinetic Parameters**

| | N | Half-life | $C_{max}$ | $AUC_{0\text{-}inf}$ | Vd | CL |
|---|---|---|---|---|---|---|
| | | (min) | (ng/mL) | (ng•min/mL) | (L/kg) | (mL/min/kg) |
| DL1 D1 | 4 | 28.2 (8.5) | 47.7 (28.7) | 1361 (609) | 2.74 (161) | 65.2 (30.9) |
| DL1 D8 | 4 | 19.2 (14.6) | 36.5 (18.3) | 1009 (392) | 2.12 (1.66) | 82.3 (28.5) |
| DL2 D1 | 11 | 40.5 (17.2) | 98.7 (25.3) | 3031 (686) | 3.00 (1.27) | 51.9 (11.7) |
| DL2 D8 | 11 | 44.9 (19.0) | 102.7 (25.6) | 2918 (599) | 3.44 (1.59) | 53.6 (11.9) |
| | *P* D1 vs D8 | 0.731 | 0.847 | 0.639 | 0.762 | 0.639 |

Mean Standard Deviation (SD) pharmacokinetic parameters of decitabine per dose level on Day 1 (DAC alone), and Day 8 (DAC with TMZ) derived by non-compartmental analysis.

**Table 7. Temozolomide Pharmacodynamic Parameters**

| | N | Ka | Vd/F | CL/F | Half-life | $C_{max}$ |
|---|---|---|---|---|---|---|
| | | | (L) | (L/h) | (h) | (µg/mL) |
| Observed | 14 | 3.13 (2.0) | 27.3 (7.2) | 11.7 (2.5) | 1.60 (0.12) | 5.29 (1.37) |
| Predicted | 14 | - | 32.1 (3.6) | 11.6 (1.6) | 1.93 (0.11) | - |
| | *P* D8 vs predicted | - | 0.0017 | 0.855 | 0.00012 | - |

Mean (Standard Deviation, SD) pharmacokinetic parameters of temozolomide on Day 8 as observed and calculated with a 1 compartment population model, and as predicted based on BSA and sex according to Ostermann et al. $C_{max}$ was visually determined from the raw data.

**Table 8. Patients with Tumor Biopsies and Paired PBMCs**

| | Patient 3 | Patient 5 | Patient 19 | Patient 25 | Patient 30 | Patient 32 | Patient 33 | Patient 39 |
|---|---|---|---|---|---|---|---|---|
| **Day 1** | | | | | | | | |
| Tumor | X | X | X | X | X | X | X | X |
| PBMC | X | X | X | X | X | X | X | X |
| | | | | | | | | |
| **Day 8** | | | | | | | | |
| Tumor | X | X | X | X | | | | X |
| PBMC | X | X | X | X | X | X | X | X |
| | | | | | | | | |
| **Day 15** | | | | | | | | |
| Tumor | | X | X | X | | | | X |
| PBMC | X | X | X | X | X | X | X | X |
| | | | | | | | | |
| **Day 29** | | | | | | | | |
| Tumor | | X | X | | | | X | X |
| PBMC | X | X | X | X | X | X | X | X |

**Table 9. Changes in Global Methylation**

| Patient # | | 3 | 5 | 19 | 25 | 30 | 33 | 39 |
|---|---|---|---|---|---|---|---|---|
| | Methylation Status | No. (%) | No. (%) | No. (%) | No. (%) | No. (%) | No. (%) | No. (%) |
| **Days 1 vs 8** | | | | | | | | |
| Tumor | Decrease | 666 (2.4) | 938 (3.4) | 1771 (6.4) | 958 (3.5) | | | 649 (2.4) |
| | Increase | 636 (2.3) | 762 (2.8) | 486 (1.8) | 977 (3.5) | | | 359 (1.3) |
| **Days 1 vs 15** | | | | | | | | |
| Tumor | Decrease | | 1570 (5.7) | 1392 (5.1) | 1197 (4.3) | | | 1242 (4.5) |
| | Increase | | 822 (3.0) | 421 (1.5) | 833 (3.0) | | | 596 (2.2) |
| PBMC | Decrease | | 1 (0.01) | 3259 (11.8) | 233 (0.84) | | | |
| | Increase | | 1 (0.01) | 4 (0.02) | 15 (0.05) | | | |
| **Days 1 vs 29** | | | | | | | | |
| Tumor | Decrease | | 2160 (7.8) | 1271 (4.6) | | | 2911 (10.6) | 784 (2.8) |
| | Increase | | 695 (2.5) | 567 (2.1) | | | 1802 (6.5) | 198 (0.7) |
| PBMC | Decrease | | 1 (0.01) | 274 (1.0) | | 84 (0.3) | 3 (0.01) | |
| | Increase | | 0 (0.0) | 2843 (10.3) | | 20 (0.07) | 9 (0.03) | |

**Table 10. Changes in Gene Expression**

| Patient # | | 3 | 5 | 19 | 25 | 30 | 33 | 39 |
|---|---|---|---|---|---|---|---|---|
| | Gene Expression | No. (%) | No. (%) | No. (%) | No. (%) | No. (%) | No. (%) | No. (%) |
| **Days 1 vs 8** | | | | | | | | |
| Tumor | Decrease | 1605 (5.1) | 77 (0.2) | 444 (1.4) | 6575 (20.9) | | | 5890 (18.7) |
| | Increase | 77 (0.2) | 630 (2.0) | 77 (0.2) | 20 (0.1) | | | 11 (0.04) |
| | | | | | | | | |
| **Days 1 vs 15** | | | | | | | | |
| Tumor | Decrease | | 340 (1.1) | 928 (2.9) | 2281 (7.2) | | | 3290 (10.5) |
| | Increase | | 372 (1.2) | 73 (0.2) | 7 (0.02) | | | 106 (0.3) |
| | | | | | | | | |
| **Days 1 vs 29** | | | | | | | | |
| Tumor | Decrease | | 61 (0.2) | 1675 (5.3) | | | 489 (1.6) | 62 (0.2) |
| | Increase | | 564 (1.8) | 17 (0.1) | | | 116 (0.4) | 557 (1.8) |

# 5.0 CONCLUSION AND FUTURE DIRECTIONS

In this dissertation I have examined the role of epigenetic regulation on chemotherapy resistance in metastatic melanoma. My findings can be summarized as follows: 1) the promoter methylation and gene expression of *MLH1* are important predictors of survival and response to alkylator-based chemotherapy in metastatic melanoma; 2) a Phase I/II clinical trial was conducted with DAC in combination with extended-schedule TMZ administered for patients with metastatic melanoma. A recommended Phase II dose of the combination was established that led to improved clinical outcomes including response rate, clinical benefit rate, median PFS and median OS; 3) The observed improvement in clinical outcome was shown to not be secondary to increased exposure of either drug and was not clearly related to changes in the methylation or expression of DNA repair genes.

My findings from the retrospective study will require several levels of validation: 1) validation of the high-throughput signals with RT-PCR and possibly with immunohistochemistry for protein expression in the tumor tissues examined; 2) validation in a separate cohort, preferably not treated at our institution- discussions in that regard are already underway with potential collaborators; 3) prospective validation in a cohort of patients being treated with temozolomide or dacarbazine where clinical data can be collected real-time significantly reducing the sources of bias inherent to retrospective studies. It is conceivable that prospective clinical trials can be designed that incorporate assessment of MLH1 gene expression and

promoter methylation as either an inclusion criterion or a stratification parameter. In the future, it is planned to continue examining novel integrative models that will allow the concurrent analysis of gene expression and promoter methylation at the whole genome level. Our current data will serve as a platform for such modeling approaches with plans to incorporate BRAF mutational testing into chemotherapy response prediction models using the novel methodology of entromics (this proposal has received pilot funding from the Clinical Translational Science Institute (CTSI) for the Basic to Clinical Collaborative Research Pilot Program (BaCCOR) in collaboration with Dr. Petr Pancoska).

These findings in the Phase I/II trial of decitabine in combination with temozolomide for patients with metastatic melanoma are quite promising. I plan to propose a randomized controlled Phase II or III clinical trial to confirm our findings in patients with wild-type BRAF or those with BRAF mutations that have progressed on BRAF inhibitors. I am considering the incorporation of testing for MLH1 expression and methylation in the study design with the goal of validating the predictive value of MLH1 in chemotherapy response as well as move forward patient selection methods as means to improve outcomes in patients with metastatic melanoma. Finally, I plan to propose a Phase I clinical trial that adds PARP inhibition to the dual modulation strategy of DAC + TMZ, which we have termed “triple modulation of DNA repair”. This Phase I trial was already proposed to NCI-CTEP and received pilot funding from the Skin SPORE as a Developmental Research Project (DRP).

## BIBLIOGRAPHY


1. Skin Cancer. American Cancer Society, 2010. (Accessed 04/10/2011, 2011, at http://www.cancer.org/.)

2. Rigel DS. Malignant melanoma: perspectives on incidence and its effects on awareness, diagnosis, and treatment. CA Cancer J Clin 1996;46:195-8.

3. Cummins DL, Cummins JM, Pantle H, Silverman MA, Leonard AL, Chanmugam A. Cutaneous malignant melanoma. Mayo Clin Proc 2006;81:500-7.

4. MacKie RM, Hauschild A, Eggermont AM. Epidemiology of invasive cutaneous melanoma. Ann Oncol 2009;20 Suppl 6:vi1-7.

5. Welch HG, Woloshin S, Schwartz LM. Skin biopsy rates and incidence of melanoma: population based ecological study. BMJ 2005;331:481.

6. Jemal A, Devesa SS, Hartge P, Tucker MA. Recent trends in cutaneous melanoma incidence among whites in the United States. J Natl Cancer Inst 2001;93:678-83.

7. Beddingfield FC, 3rd. The melanoma epidemic: res ipsa loquitur. Oncologist 2003;8:459-65.

8. Tsao H, Rogers GS, Sober AJ. An estimate of the annual direct cost of treating cutaneous melanoma. J Am Acad Dermatol 1998;38:669-80.

9. Seidler AM, Pennie ML, Veledar E, Culler SD, Chen SC. Economic burden of melanoma in the elderly population: population-based analysis of the Surveillance, Epidemiology, and End Results (SEER)--Medicare data. Arch Dermatol 2010;146:249-56.

10. Kirkwood JM, Manola J, Ibrahim J, Sondak V, Ernstoff MS, Rao U. A pooled analysis of eastern cooperative oncology group and intergroup trials of adjuvant high-dose interferon for melanoma. Clin Cancer Res 2004;10:1670-7.

11. Barth A, Wanek LA, Morton DL. Prognostic factors in 1,521 melanoma patients with distant metastases. J Am Coll Surg 1995;181:193-201.

12. Tawbi HA, Kirkwood JM. Management of metastatic melanoma. Semin Oncol 2007;34:532-45.

13. Yang AS, Chapman PB. The history and future of chemotherapy for melanoma. Hematol Oncol Clin North Am 2009;23:583-97, x.

14. Agarwala SS, Kirkwood JM. Temozolomide, a novel alkylating agent with activity in the central nervous system, may improve the treatment of advanced metastatic melanoma. Oncologist 2000;5:144-51.

15. Middleton MR, Lorigan P, Owen J, et al. A randomized phase III study comparing dacarbazine, BCNU, cisplatin and tamoxifen with dacarbazine and interferon in advanced melanoma. Br J Cancer 2000;82:1158-62.

16. Atkins MB. Interleukin-2: clinical applications. Semin Oncol 2002;29:12-7.

17. Hodi FS, O'Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 2010;363:711-23.

18. Flaherty KT, Puzanov I, Kim KB, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. N Engl J Med 2010;363:809-19.

19. Liu L, Gerson SL. Targeted modulation of MGMT: clinical implications. Clin Cancer Res 2006;12:328-31.

20. Tawbi H, Tarhini A, Moschos S, Radkowski R, Shipe-Spotloe J, Kirkwood J. Phase I trial of Patrin (Lomeguatrib) combined with dacarbazine (DTIC) for treatment of patients with melanoma and other solid tumors: Initial Results. Journal of Clinical Oncology 2006;24:8016.

21. Barvaux VA, Ranson M, Brown R, McElhinney RS, McMurry TB, Margison GP. Dual repair modulation reverses Temozolomide resistance in vitro. Mol Cancer Ther 2004;3:123-7.

22. Horton TM, Jenkins G, Pati D, et al. Poly(ADP-ribose) polymerase inhibitor ABT-888 potentiates the cytotoxic activity of temozolomide in leukemia cells: influence of mismatch repair status and O6-methylguanine-DNA methyltransferase activity. Mol Cancer Ther 2009;8:2232-42.

23. Palma JP, Rodriguez LE, Bontcheva-Diaz VD, et al. The PARP inhibitor, ABT-888 potentiates temozolomide: correlation with drug levels and reduction in PARP activity in vivo. Anticancer Res 2008;28:2625-35.

24. Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. Nature 2004;429:457-63.

25. Jones PA, Takai D. The role of DNA methylation in mammalian epigenetics. Science 2001;293:1068-70.

26. Kaneda M, Okano M, Hata K, et al. Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. Nature 2004;429:900-3.

27. Zhu WG, Otterson GA. The interaction of histone deacetylase inhibitors and DNA methyltransferase inhibitors in the treatment of human cancer cells. Curr Med Chem Anticancer Agents 2003;3:187-99.

28. Rothhammer T, Bosserhoff AK. Epigenetic events in malignant melanoma. Pigment Cell Res 2007;20:92-111.

29. Takai D, Jones PA. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc Natl Acad Sci U S A 2002;99:3740-5.

30. Lyko F, Brown R. DNA methyltransferase inhibitors and the development of epigenetic cancer therapies. J Natl Cancer Inst 2005;97:1498-506.

31. Arthur C. Decitabine. MGI Pharma Inc/SuperGen Inc. IDrugs 2004;7:1113-32.

32. Issa JP, Garcia-Manero G, Giles FJ, et al. Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2'-deoxycytidine (decitabine) in hematopoietic malignancies. Blood 2004;103:1635-40.

33. Middleton MR, Grob JJ, Aaronson N, et al. Randomized phase III study of temozolomide versus dacarbazine in the treatment of patients with advanced metastatic malignant melanoma. J Clin Oncol 2000;18:158-66.

34. Korn EL, Liu PY, Lee SJ, et al. Meta-analysis of phase II cooperative group trials in metastatic stage IV melanoma to determine progression-free and overall survival benchmarks for future phase II trials. J Clin Oncol 2008;26:527-34.

35. Serrone L, Zeuli M, Sega FM, Cognetti F. Dacarbazine-based chemotherapy for metastatic melanoma: thirty-year experience overview. J Exp Clin Cancer Res 2000;19:21-34.

36. Stevens MF, Hickman JA, Langdon SP, et al. Antitumor activity and pharmacokinetics in mice of 8-carbamoyl-3-methyl-imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (CCRG 81045; M & B 39831), a novel drug with potential as an alternative to dacarbazine. Cancer Res 1987;47:5846-52.

37. Tawbi HA, Buch SC. Chemotherapy resistance abrogation in metastatic melanoma. Clin Adv Hematol Oncol 2010;8:259-66.

38. Kaina B, Ochs K, Grosch S, et al. BER, MGMT, and MMR in defense against alkylation-induced genotoxicity and apoptosis. Prog Nucleic Acid Res Mol Biol 2001;68:41-54.

39. Kaina B, Christmann M, Naumann S, Roos WP. MGMT: key node in the battle against genotoxicity, carcinogenicity and apoptosis induced by alkylating agents. DNA Repair (Amst) 2007;6:1079-99.

40. Alvino E, Castiglia D, Caporali S, et al. A single cycle of treatment with temozolomide, alone or combined with O(6)-benzylguanine, induces strong chemoresistance in melanoma cell clones in vitro: role of O(6)-methylguanine-DNA methyltransferase and the mismatch repair system. Int J Oncol 2006;29:785-97.

41. Roos WP, Batista LF, Naumann SC, et al. Apoptosis in malignant glioma cells triggered by the temozolomide-induced DNA lesion O6-methylguanine. Oncogene 2007;26:186-97.

42. Boeckmann L, Schirmer M, Rosenberger A, et al. Effect of DNA repair host factors on temozolomide or dacarbazine melanoma treatment in Caucasians. Pharmacogenet Genomics 2009;19:760-9.

43. Boeckmann L, Thoms KM, Gutzmer R, et al. Modulation of the efficacy of temozolomide and dacarbazine melanoma treatment by DNA-repair factors in vivo and in vitro. Int J Clin Pharmacol Ther 2009;47:33-5.

44. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57-70.

45. Brown R, Strathdee G. Epigenomics and epigenetic therapy of cancer. Trends Mol Med 2002;8:S43-8.

46. Brown R, Plumb JA. Demethylation of DNA by decitabine in cancer chemotherapy. Expert Rev Anticancer Ther 2004;4:501-10.

47. Steele N, Finn P, Brown R, Plumb JA. Combined inhibition of DNA methylation and histone acetylation enhances gene re-expression and drug sensitivity in vivo. Br J Cancer 2009;100:758-63.

48. Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. Nat Rev Genet 2007;8:286-98.

49. Middleton MR, Lunn JM, Morris C, et al. O6-methylguanine-DNA methyltransferase in pretreatment tumour biopsies as a predictor of response to temozolomide in melanoma. Br J Cancer 1998;78:1199-202.

50. Hegi ME, Diserens AC, Gorlia T, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med 2005;352:997-1003.

51. Fink D, Aebi S, Howell SB. The role of DNA mismatch repair in drug resistance. Clin Cancer Res 1998;4:1-6.

52. Colella G, Marchini S, D'Incalci M, Brown R, Broggini M. Mismatch repair deficiency is associated with resistance to DNA minor groove alkylating agents. Br J Cancer 1999;80:338-43.

53. Yount RJ, Vries JK, Councill CD. The Medical Archival System - an Information-Retrieval System Based on Distributed Parallel Processing. Inform Process Manag 1991;27:379-89.

54. . (Accessed at http://www.illumina.com/downloads/InfMethylation_AppNote.pdf.)

55. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A 2001;98:5116-21.

56. Korn EL, Troendle JF, McShane LM, Simon R. Controlling the number of false discoveries: application to high-dimensional genomic data. J Stat Plan Infer 2004;124:379-98.

57. Wang Y, Tetko IV, Hall MA, et al. Gene selection from microarray data for cancer classification--a machine learning approach. Comput Biol Chem 2005;29:37-46.

58. Ogino S, Kawasaki T, Kirkner GJ, Loda M, Fuchs CS. CpG island methylator phenotype-low (CIMP-low) in colorectal cancer: possible associations with male sex and KRAS mutations. J Mol Diagn 2006;8:582-8.

59. Bittner M, Meltzer P, Chen Y, et al. Molecular classification of cutaneous malignant melanoma by gene expression profiling. Nature 2000;406:536-40.

60. Pavey S, Johansson P, Packer L, et al. Microarray expression profiling in melanoma reveals a BRAF mutation signature. Oncogene 2004;23:4060-7.

61. Haqq C, Nosrati M, Sudilovsky D, et al. The gene expression signatures of melanoma progression. Proc Natl Acad Sci U S A 2005;102:6092-7.

62. Potti A, Dressman HK, Bild A, et al. Genomic signatures to guide the use of chemotherapeutics. Nat Med 2006;12:1294-300.

63. Staunton JE, Slonim DK, Coller HA, et al. Chemosensitivity prediction by transcriptional profiling. Proc Natl Acad Sci U S A 2001;98:10787-92.

64. Scherf U, Ross DT, Waltham M, et al. A gene expression database for the molecular pharmacology of cancer. Nat Genet 2000;24:236-44.

65. Augustine CK, Yoo JS, Potti A, et al. Genomic and molecular profiling predicts response to temozolomide in melanoma. Clin Cancer Res 2009;15:502-10.

66. Baylin SB, Esteller M, Rountree MR, Bachman KE, Schuebel K, Herman JG. Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. Hum Mol Genet 2001;10:687-92.

67. Teodoridis JM, Hall J, Marsh S, et al. CpG island methylation of DNA damage response genes in advanced ovarian cancer. Cancer Res 2005;65:8961-7.

68. Jones PA. Overview of cancer epigenetics. Semin Hematol 2005;42:S3-8.

69. Issa JP. DNA methylation in the treatment of hematologic malignancies. Clin Adv Hematol Oncol 2005;3:684-6.

70. Agrelo R, Cheng WH, Setien F, et al. Epigenetic inactivation of the premature aging Werner syndrome gene in human cancer. Proc Natl Acad Sci U S A 2006;103:8822-7.

71. Plumb JA, Strathdee G, Sludden J, Kaye SB, Brown R. Reversal of drug resistance in human tumor xenografts by 2'-deoxy-5-azacytidine-induced demethylation of the hMLH1 gene promoter. Cancer Res 2000;60:6039-44.

72. Esteller M. Epigenetic lesions causing genetic lesions in human cancer: promoter hypermethylation of DNA repair genes. Eur J Cancer 2000;36:2294-300.

73. Satoh A, Toyota M, Itoh F, et al. Epigenetic inactivation of CHFR and sensitivity to microtubule inhibitors in gastric cancer. Cancer Res 2003;63:8606-13.

74. Shen L, Kondo Y, Ahmed S, et al. Drug sensitivity prediction by CpG island methylation profile in the NCI-60 cancer cell line panel. Cancer Res 2007;67:11335-43.

75. Halaban R, Krauthammer M, Pelizzola M, et al. Integrative analysis of epigenetic modulation in melanoma cell response to decitabine: clinical implications. PLoS One 2009;4:e4563.

76. Rietschel P, Wolchok JD, Krown S, et al. Phase II study of extended-dose temozolomide in patients with melanoma. J Clin Oncol 2008;26:2299-304.

77. Reu FJ, Leaman DW, Maitra RR, et al. Expression of RASSF1A, an epigenetically silenced tumor suppressor, overcomes resistance to apoptosis induction by interferons. Cancer Res 2006;66:2785-93.

78. Christmann M, Pick M, Lage H, Schadendorf D, Kaina B. Acquired resistance of melanoma cells to the antineoplastic agent fotemustine is caused by reactivation of the DNA repair gene MGMT. Int J Cancer 2001;92:123-9.

79. Mori T, O'Day SJ, Umetani N, et al. Predictive utility of circulating methylated DNA in serum of melanoma patients receiving biochemotherapy. J Clin Oncol 2005;23:9351-8.

80. Watson AJ, Middleton MR, McGown G, et al. O(6)-methylguanine-DNA methyltransferase depletion and DNA damage in patients with melanoma treated with temozolomide alone or with lomeguatrib. Br J Cancer 2009;100:1250-6.

81. Hassel JC, Sucker A, Edler L, et al. MGMT gene promoter methylation correlates with tolerance of temozolomide treatment in melanoma but not with clinical outcome. Br J Cancer 2010;103:820-6.

82. Karran P, Macpherson P, Ceccotti S, Dogliotti E, Griffin S, Bignami M. O6-methylguanine residues elicit DNA repair synthesis by human cell extracts. J Biol Chem 1993;268:15878-86.

83. Castiglia D, Pagani E, Alvino E, et al. Biallelic somatic inactivation of the mismatch repair gene MLH1 in a primary skin melanoma. Genes Chromosomes Cancer 2003;37:165-75.

84. Strathdee G, MacKean MJ, Illand M, Brown R. A role for methylation of the hMLH1 promoter in loss of hMLH1 expression and drug resistance in ovarian cancer. Oncogene 1999;18:2335-41.

85. Gifford G, Paul J, Vasey PA, Kaye SB, Brown R. The acquisition of hMLH1 methylation in plasma DNA after chemotherapy predicts poor survival for ovarian cancer patients. Clin Cancer Res 2004;10:4420-6.

86. Samimi G, Fink D, Varki NM, et al. Analysis of MLH1 and MSH2 expression in ovarian cancer before and after platinum drug-based chemotherapy. Clin Cancer Res 2000;6:1415-21.

87. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. CA Cancer J Clin 2010;60:2 77-300.

88. Bedikian AY, Millward M, Pehamberger H, et al. Bcl-2 antisense (oblimersen sodium) plus dacarbazine in patients with advanced melanoma: the Oblimersen Melanoma Study Group. J Clin Oncol 2006;24:4738-45.

89. Schadendorf D, Ugurel S, Schuler-Thurner B, et al. Dacarbazine (DTIC) versus vaccination with autologous peptide-pulsed dendritic cells (DC) in first-line treatment of patients with metastatic melanoma: a randomized phase III trial of the DC study group of the DeCOG. Ann Oncol 2006;17:563-70.

90. Patel P, Suciu S, Mortier L, et al. Extended Schedule, Escalated Dose Temozolomide Versus Dacarbazine In Stage Iv Malignant Melanoma: Final Results Of The Randomised Phase Iii Study (Eortc 18032). In: European Society of Medical Oncology Medical Congress; 2008; Stockholm; 2008. p. viii1-viii4.

91. Gerson SL. MGMT: its role in cancer aetiology and cancer therapeutics. Nat Rev Cancer 2004;4:296-307.

92. Gajewski TF, Sosman J, Gerson SL, et al. Phase II trial of the O6-alkylguanine DNA alkyltransferase inhibitor O6-benzylguanine and 1,3-bis(2-chloroethyl)-1-nitrosourea in advanced melanoma. Clin Cancer Res 2005;11:7861-5.

93. Spiro TP, Gerson SL, Liu L, et al. O6-benzylguanine: a clinical trial establishing the biochemical modulatory dose in tumor tissue for alkyltransferase-directed DNA repair. Cancer Res 1999;59:2402-10.

94. Schilsky RL, Dolan ME, Bertucci D, et al. Phase I clinical and pharmacological study of O6-benzylguanine followed by carmustine in patients with advanced cancer. Clin Cancer Res 2000;6:3025-31.

95. Schold SC, Jr., Kokkinakis DM, Chang SM, et al. O6-benzylguanine suppression of O6-alkylguanine-DNA alkyltransferase in anaplastic gliomas. Neuro Oncol 2004;6:28-32.

96. Friedman HS, Pluda J, Quinn JA, et al. Phase I trial of carmustine plus O6-benzylguanine for patients with recurrent or progressive malignant glioma. J Clin Oncol 2000;18:3522-8.

97. Quinn JA, Desjardins A, Weingart J, et al. Phase I trial of temozolomide plus O6-benzylguanine for patients with recurrent or progressive malignant glioma. J Clin Oncol 2005;23:7178-87.

98. Weingart J, Grossman SA, Carson KA, et al. Phase I trial of polifeprosan 20 with carmustine implant plus continuous infusion of intravenous O6-benzylguanine in adults with recurrent malignant glioma: new approaches to brain tumor therapy CNS consortium trial. J Clin Oncol 2007;25:399-404.

99. Warren KE, Aikin AA, Libucha M, et al. Phase I study of O6-benzylguanine and temozolomide administered daily for 5 days to pediatric patients with solid tumors. J Clin Oncol 2005;23:7646-53.

100. Quinn JA, Jiang SX, Reardon DA, et al. Phase II trial of temozolomide plus o6-benzylguanine in adults with recurrent, temozolomide-resistant malignant glioma. J Clin Oncol 2009;27:1262-7.

101. Ryan CW, Dolan ME, Brockstein BB, et al. A phase II trial of O6-benzylguanine and carmustine in patients with advanced soft tissue sarcoma. Cancer Chemother Pharmacol 2006;58:634-9.

102. Batts ED, Maisel C, Kane D, et al. O6-benzylguanine and BCNU in multiple myeloma: a phase II trial. Cancer Chemother Pharmacol 2007;60:415-21.

103. Tolcher AW, Gerson SL, Denis L, et al. Marked inactivation of O6-alkylguanine-DNA alkyltransferase activity with protracted temozolomide schedules. Br J Cancer 2003;88:1004-11.

104. Sobol RW, Tawbi H, Jukic D, Mule K, Mascari R, Kirkwood J. Mismatch repair (MMR) and base excision repair (BER) protein expression correlates with clinical response to dacarbazine (DTIC)/temozolomide (TMZ) therapy of patients with metastatic melanoma. . Journal of Clinical Oncology 2006;24:8015.

105. Decitabine (Dacogen) for myelodysplastic syndromes. Med Lett Drugs Ther 2006;48:91-2.

106. Appleton K, Mackay HJ, Judson I, et al. Phase I and pharmacodynamic trial of the DNA methyltransferase inhibitor decitabine and carboplatin in solid tumors. J Clin Oncol 2007;25:4603-9.

107. Gollob JA, Sciambi CJ, Peterson BL, et al. Phase I trial of sequential low-dose 5-aza-2'-deoxycytidine plus high-dose intravenous bolus interleukin-2 in patients with melanoma or renal cell carcinoma. Clin Cancer Res 2006;12:4619-27.

108. Loeb LA, Loeb KR, Anderson JP. Multiple mutations and cancer. Proc Natl Acad Sci U S A 2003;100:776-81.

109. Kantarjian H, Issa JP, Rosenfeld CS, et al. Decitabine improves patient outcomes in myelodysplastic syndromes: results of a phase III randomized study. Cancer 2006;106:1794-803.

110. Kim H, Likhari P, Parker D, et al. High-performance liquid chromatographic analysis and stability of anti-tumor agent temozolomide in human plasma. J Pharm Biomed Anal 2001;24:461-8.

111. D'Argenio DZ SA, Wang X. ADAPT 5 User's guide: Pharmacokinetic/Pharmacodynamic Systems Analysis Software. Los Angeles: Biomedical Simulations Resource; 2009.

112. Ostermann S, Csajka C, Buclin T, et al. Plasma and cerebrospinal fluid population pharmacokinetics of temozolomide in malignant glioma patients. Clin Cancer Res 2004;10:3728-36.

113. Stewart DJ, Issa JP, Kurzrock R, et al. Decitabine effect on tumor global DNA methylation and other parameters in a phase I trial in refractory solid tumors and lymphomas. Clin Cancer Res 2009;15:3881-8.

114. Schrump DS, Fischette MR, Nguyen DM, et al. Phase I study of decitabine-mediated gene expression in patients with cancers involving the lungs, esophagus, or pleura. Clin Cancer Res 2006;12:5777-85.

115. Koshy M, Dorn L, Bressler L, et al. 2-deoxy 5-azacytidine and fetal hemoglobin induction in sickle cell anemia. Blood 2000;96:2379-84.

116. Cashen AF, Shah AK, Todt L, Fisher N, DiPersio J. Pharmacokinetics of decitabine administered as a 3-h infusion to patients with acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS). Cancer Chemother Pharmacol 2008;61:759-66.