**Epigenetic Targeting of Platinum Resistant Testicular Cancer**

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**Abstract**

The involvement of epigenetic aberrations in the development and progression of tumors is now well established. However, little is known of the epigenetic alterations in testicular cancer and particularly in platinum refractory germ cell tumors. Germ cell derived testicular cancers, as compared to somatic tumors, appear to have a unique epigenetic profile that features more extensive DNA hypomethylation. Emerging data from clinical specimens suggest that epigenetic aberrations, especially DNA hypermethylation, can contribute to chemotherapy resistance and poor clinical outcomes in testicular germ cell tumors. Recent data indicate that testicular cancer cells, even those resistant to platinum, are highly sensitive to low doses of demethylating agents. Based on these promising preclinical studies, we suggest that DNA methylation inhibitors in combination with chemotherapeutic agents may offer a path to overcome acquired drug resistance in testicular cancer, laying the foundation and rationale for testing this class of epigenetic drugs in the clinical setting. In this mini-review we provide a brief overview of the promise of DNA methylation therapy to treat patients with refractory cancer of the testes.

**Keywords**

Testicular cancer; germ cell tumor; embryonal carcinoma; cisplatin resistance; epigenetics; DNA methyltransferase; decitabine; SGI-110

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**INTRODUCTION**

Testicular cancer is the most common cancer in men aged 15 to 40 years in the United States, Canada, and Europe [1]. Ninety-five percent of testicular neoplasms are germ cell tumors (GCTs). GCTs consist of two histologically distinct subtypes: seminomas (40%) and non-seminomas (60%). Non-seminomatous GCTs are further divided into embryonal carcinoma (EC), teratoma, yolk sac tumor (YST), and choriocarcinoma [2]. The incidence of testicular GCTs has been increasing in the last 30 years. Since no strong behavioral or
environmental risk factors have been identified, treatment and cure, rather than screening and prevention, is paramount to fighting this type of cancer [1].

The most important development in the treatment of advanced testicular cancer was the discovery of cisplatin [3]. Cisplatin was established as the essential component for the treatment of advanced disease and marked the beginning of the modern era of chemotherapy for GCTs [4]. This discovery increased the 5-year survival rate of patients with metastatic GCTs from 5% to over 80% [5, 6]. In 1997, the International Germ Cell Cancer Collaborative Group (IGCCCG) published an analysis from almost 6000 patients treated for metastatic GCTs. The IGCCCG risk stratification system classified patients with metastatic GCTs at the onset of diagnosis into three groups: good, intermediate and poor risk with cure rates of 90%, 75% and 50%, respectively [7]. The standard therapy for these patients is cisplatin-based combination chemotherapy. Patients who relapse after first line chemotherapy have approximately a 50% chance of achieving a cure through the use of salvage chemotherapy. This consists of either high-dose chemotherapy (HDCT) followed by a peripheral stem cell transplant or traditional chemotherapy [8]. Nearly all patients progressing after HDCT will ultimately die from progressive disease [6, 9]. Patients with platinum refractory disease are defined as progressing while on cisplatin based chemotherapy or within 6 weeks after completing chemotherapy or after HDCT. Multiple studies from Indiana University have shown that further chemotherapy after HDCT results in a response rate of less than 20%, with a median survival time of 8 months. All deaths occurred within 12 months of HDCT [10]. In addition, seven phase II clinical trials evaluated single-agent activity in 90 refractory GCT patients at Memorial Sloan Kettering Cancer Center. These trials showed a median progression free survival (PFS) of 4 weeks and a median overall survival (OS) of 4 months [9]. The failure of these trials highlights the need to explore other pathways and targets to combat the refractory form of this disease. Morbidity and mortality in this age group occur during the most productive years of a patient’s life. An average of more than 35 years of life is lost when a patient dies from this disease, well over a decade longer than any other adult malignancy.

**EPIGENETIC ALTERATIONS IN TESTICULAR CANCER**

Epigenetic features of neoplasms are being explored as possible novel therapeutic options. Epigenetic alterations are defined as changes in gene expression without alteration of the DNA sequence. This can include DNA methylation, histone modification, nucleosome repositioning, and posttranscriptional gene regulation by micro-RNA (miRNA) [11, 12]. In recent years, DNA methylation has increasingly become a focus of study in GCTs and will be the topic of this review.

DNA methylation can be defined as the addition of a methyl moiety to the cytosine-5 position within the context of a CpG dinucleotide, mediated by DNA methyltransferases (DNMTs) [11]. DNA methylation is an important regulator of gene transcription. Its role in carcinogenesis and resistance to therapy has been a topic of considerable recent interest. Hypermethylation represses transcription of CpG-rich promoter regions of tumor suppressor genes leading to gene silencing [13]. Hypomethylation is associated with gene activation whereas hypermethylation is associated with gene inactivation. Epigenetic regulation of
gene expression is complex and also involves posttranslational modifications of histones which is in part mediated by recruitment of DNA binding proteins to methylated CpGs. The net effect is a regional condensation of chromatin structure. A unique feature of GCTs that may explain their exquisite sensitivity to platinum therapies compared to other solid somatic tumors is global CpG hypomethylation [14, 15].

Seminomas have very little DNA methylation compared to normal cells and somatic tumors while non-seminomas appear to have an intermediate level of DNA methylation [16-20] (Fig. 1). Candidate approaches found certain tumor suppressors including RASSF1A, MGMT, HIC1, APOLD1, PCDH10 and RAG1 to be hypermethylated in GCTs and reactivation of gene expression occurred in GCT cell lines treated with demethylating agents [18, 21]. Recent detailed genome wide methylation studies in seminoma and nonseminoma cell lines and patient samples confirm the concept that GCTs may have originated from an erased embryonic germ cell, with decreased methylation of CpG islands, imprinted genes and repetitive elements [22, 23]. The hypo-methylation of seminomas correlates with hypersensitivity to chemotherapy. Within nonseminoma there is increased methylation associated with differentiation from pluripotent EC to teratoma that correlates with sensitivity to cisplatin [19, 21, 24, 25].

**MiRNA IN GCTs**

More recently discovered epigenetic changes in cancer involve miRNA. These small (19-22 nucleotides in length) non-coding RNAs regulate protein expression through posttranscriptional down-regulation and RNA silencing [26]. Interestingly, DNA methylation can regulate miRNA expression as shown in a study where a hypomethylating agent increased miRNA production [27]. Recently an important role for miRNAs in GCTs has been proposed. A variety of miRNAs have been found to be elevated in GCT patient serum including miR-302a-3p, 302b-3p, 302c-3p, 367-3p, 371a-3p, 372-3p and 373-3p [28]. In particular, miR-371a-3p was discovered to be both sensitive (84.7%) and specific (99%) when used as a serum tumor marker of GCTs while non-testicular malignancies had levels of miR-371a-3p similar to control patients [28]. In GCT patients miR-371a-3p has been shown to increase with advancing disease and tumor bulk as well as decrease after tumor resection [28]. Importantly, miR-371a-3p outperformed current tumor markers human chorionic gonadotropin (HCG) and alpha-fetoprotein (AFP) in a study of 76 GCT patients [29]. This finding has high clinical significance as HCG and AFP are only elevated in 60% of GCT patients [30]. These studies show that epigenetic features of GCTs such as miRNAs could provide future biomarkers for this disease.

**A MECHANISM FOR PLATINUM RESISTANCE IN TESTICULAR CANCER**

Epigenetic silencing, such as hypermethylation, may play a role in resistance to platinum in refractory GCTs [18, 21, 31]. Cisplatin acts by forming platinum-DNA lesions in dividing cells, forcing them to undergo DNA repair or apoptosis. Hypomethylated DNA is generally thought to be more active, or “open”, allowing for easier integration of platinum into DNA leading to more cellular damage and apoptosis. When genes become hypermethylated this
access is decreased, possibly leading to resistance. Hypermethylation is a reversible phenomenon and thus can be targeted [32].

Platinum refractory GCTs have been associated with hypermethylation in the promoter regions of tumor suppressor genes. Hypermethylation of RASSF1A and H1C1, was found to be associated with resistance in GCTs [21]. Additionally, cisplatin treatment of GCTs was found to induce de novo promoter hypermethylation in vivo [21]. Wermann et al. also showed a relationship between DNA methylation and chemotherapy resistance in GCTs by reporting that a cisplatin resistant seminoma was hypermethylated compared to sensitive seminoma [33]. These findings have implications as to how GCTs may become refractory to platinum based therapy.

Epigenetic silencing through methylation has been demonstrated to have similar effects to DNA mutations in tumorigenesis and chemotherapy resistance and has led to studies designed to reverse this potential mechanism in platinum refractory tumors [33]. De novo methylation of DNA involves DNMT3A and DNMT3B [35, 36]. Elevated DNMT3B levels are associated with tumor invasion in seminomatous GCT. Stage I and Stage II seminomas showed high expression of DNMT3B in 36 and 38% of tumors, respectively [37]. Stage III seminomas showed high expression of DNMT3B in 100% of tumors studied [37]. Additionally, if DNMT3B expression was not elevated, relapse occurred in only 7% of cases compared with over 24% of cases with elevated DNMT3B. Seminomas that had high expression levels of DNMT3B had a lower relapse-free survival rate [37].

PRECLINICAL EPIGENETIC DRUG STUDIES IN TESTICULAR CANCER

The nucleoside analogs 5-aza-deoxycytidine (5-aza-CdR, decitabine) and 5-aza-cytidine (5-aza-CR) are potent inhibitors of DNA methylation (Fig. 2). DNA hypomethylation is mediated through the covalent trapping of DNMTs [11, 38]. This class of therapeutic agents are known as DNA methyltransferase inhibitors (DNMTIs). Studies have shown that DNMTIs hit multiple targets including the tumor suppressor gene p53. Activation of this gene leads the cell down the DNA damage response pathway and to apoptosis in EC cells [39, 40]. We have shown that human pluripotent EC, the stem cells of nonseminoma GCTs, are highly sensitive to very low doses of decitabine, doses that are 100-1000 fold lower than those used to induce cytotoxicity of somatic cancer cells [41]. Low dose decitabine was effective in mediating global promoter demethylation, p53 target gene activation, cytotoxicity, and differentiation of EC cells [41, 42]. Low nanomolar concentrations of decitabine caused decreased cell proliferation and survival and the reexpression of genes silenced by DNA hypermethylation including MGMT, RASSF1A, and HOXA9.

Decitabine hypersensitivity was associated with markedly high expression of pluripotency-associated DNMT3B compared with somatic tumor cells [41, 42]. Notably, hypersensitivity to decitabine also occurs in EC cells resistant to cisplatin. Further, low dose decitabine can restore cisplatin sensitivity to cisplatin resistant cells [41, 42]. This finding was recently confirmed by Wermann et al. who showed that decitabine restored sensitivity to cisplatin in a refractory seminoma cell line [33]. Knockdown of DNMT3B resulted in resistance to decitabine, suggesting that decitabine sensitivity in EC is mechanistically linked to, and
dependent on, high levels of DNMT3B [41, 42]. That a variety of EC cell lines are sensitive to very low doses of decitabine with dependence on DNMT3B was also reported by Wongtrakoongate et al. [43]. Studies in ovarian cancer using a variety of DNMTIs also reported carboplatin resensitization after DNA hypomethylation [44-46].

CLINICAL EPIGENETIC STUDIES USING DEMETHYLATING AGENTS

Decitabine is approved for the treatment of myelodysplastic syndrome (MDS) and shows promise for the treatment of specific leukemias [47]. Recent experience with MDS suggests that decitabine should be given below the maximum tolerated dose and over many cycles since tumor responses usually do not occur until after the third cycle [47]. This may explain why DNA methylation inhibitors given at maximum tolerated doses incompatible with cell division were disappointing for solid tumors. In addition, recent preclinical and clinical studies have suggested that lower “biologically effective” doses of decitabine may be an effective clinical strategy to target tumor initiating cells in solid tumors [48, 49]. Two recent phase I/II studies showed that low dose DNMTI treatment could restore sensitivity to carboplatin in patients with heavily pretreated ovarian cancer and resistance to cisplatin was correlated with hypermethylation of ovarian tumor DNA [50, 51].

In a phase II trial of GCT patients who had progressed within four weeks of cisplatin based therapy and were then treated with 5-aza-cytidine, all patients had progressive disease. Fifteen of the seventeen died of progressive disease and one from sepsis [52]. However, high, cytotoxic doses (150 mg/m²) were used that are now known to be suboptimal for mediating demethylation. More recently Matei et al. conducted a phase I/II trial of a DNMTI followed by carboplatin, in ovarian cancer [50]. This study showed that sequential low dose decitabine followed by carboplatin was well tolerated. Additionally, this study demonstrated biologic response in the form of DNA-hypomethylation that correlated with reversed platinum resistance [53]. Similar data from other institutions corroborated these findings [54].

A currently ongoing phase II trial is based on a study of 17 patients with heavily pre-treated and platinum-resistant ovarian cancer. When this population is treated only with carboplatin, there was a small objective response of less than 10% [55]. However, pre-treating with a DNMTI before carboplatin administration led to a 35% objective response rate. Additionally, patients that were pre-treated with a DNMTI had a PFS of 10.2 months with over half (53%) of patients free of disease progression at 6 months. Interestingly, tumors biopsied after treatment found that patients who had PFS of more than 6 months had a greater number of demethylated genes involved in cancer pathways than patients who had PFS of less than 6 months. This suggests that demethylation with the DNMTI decitabine has an impact in mediating platinum resensitization [50]. Fu et al. showed similar findings in the same patient population with a DNMTI and carboplatin used in succession with a 13.8% overall response rate, as well as a disease control rate (partial response rate plus stable disease) of 45% [51]. From this data, a phase II study was designed to resensitize ovarian cancer to cisplatin using the novel DNMTI, SGI-110, in ovarian cancer (NCT01696032).
In addition to ovarian cancer, a combination phase I/II trial was conducted in patients with recurrent metastatic nonsmall cell lung cancer. The patients were heavily pre-treated with three lines of chemotherapy before receiving a DNMTI and a histone deacetylase inhibitor. A subset of patients then received further cytotoxic chemotherapy. Nearly 20% of the patients lived over 1 year with some living up to 4 years [48]. The expected survival of this patient population after receiving three lines of chemotherapy is 48% at 6 months [56]. The authors of this study suggest that the response may be attributable to prolonged stabilization, but they did document an unusually robust response to subsequent cytotoxic chemotherapy [48].

**SGI-110 AS A NOVEL DEMETHYLATING AGENT**

DNMTIs such as decitabine are subject to rapid degradation by hydrolytic cleavage and deamination by cytidine deaminase and are unstable after intravenous infusion limiting their potential as cancer therapeutics [57]. Decitabine has a promising dinucleotide analogue named SGI-110 (2′-deoxy-5-azacytidylyl-(3′→5′)-2′-deoxyguanosine sodium salt) (Astex pharmaceuticals Inc.) that is not subject to the same metabolism as other DNMTIs [58] (Fig. 2). Additionally, SGI-110 can be given as a subcutaneous injection allowing for a longer effective half-life and a more extended exposure window compared to intravenous infusion of decitabine [59]. This novel DNMTI was shown to achieve hypomethylation and was well tolerated in primates [60]. Additionally, in cancer xenograft models it has proved to be an effective DNA methylation inhibitor in vivo and reduced tumor growth if MDS cells [58]. In a phase I/II trial in acute myeloid leukemia and MDS patients, SGI-110 was shown to be better tolerated and demonstrated activity in patients who had progressed on decitabine [61].

Preclinical data from Indiana University showed that like decitabine, SGI-110 also re-sensitizes platinum resistant ovarian cancer cell lines [32]. Specifically, a greater than two-fold reduction in the inhibitory concentration of platinum was achieved with SGI-110 pre-treatment. Additionally, biopsies of subcutaneous ovarian cancer xenografts showed that SGI-110 treatment induced significant demethylation and subsequent transcriptional enhancement of tumor suppressor genes [32]. SGI-110 alone or in combination with carboplatin was well tolerated in non-tumor bearing mice.

Significant antitumor activity was observed with single agent SGI-110 as well as with SGI-110 given in combination with carboplatin treatment in both biweekly and daily regimens [32]. The results of this preclinical study supports the recently launched clinical trial NCT01696032 using SGI-110 in combination with carboplatin in patients with recurrent, platinum-refractory ovarian cancer [32, 62]. The study demonstrated that the combination of SGI-110 at 30 mg/m² and carboplatin at AUC 4 is well tolerated and this regimen is currently being implemented in stage 2 of this trial [62]. Further SGI-110 was shown to inhibit GCT-derived EC equally well and with a similar mode of action as compared to decitabine in vitro and in xenografts (Fig. 3 and Albany and Spinella, unpublished).
FUTURE DIRECTIONS

DNA methyltransferase inhibitors have the ability to resensitize both platinum-refractory testicular germ cell tumors and platinum-refractory ovarian cancer to platinum based chemotherapy and studies indicate that GCT cells are exquisitely sensitive to low dose DNMTI therapy [41, 42, 49, 53]. The most recent clinical trial using a DNMTI to treat refractory GCT was unsuccessful in improving outcomes. However, in this trial 5-azacytidine was used at high doses as a non-specific cytotoxic agent and not at the low doses that are optimized to inhibit DNA methylation. The trial also did not test whether DNMTI therapy could chemosensitize refractory GCTs to platinum [52]. The aforementioned preclinical trials have shown DNMTIs to have success in resensitizing platinum refractory ovarian cancer cells. The progression of this basic science discovery to a clinical trial using SGI-110 as a DNMTI to sensitize platinum-refractory ovarian cancer is currently underway (NCT01696032). With the evidence that SGI-110 also acts to demethylate refractory GCT cell lines, restoring the chemotherapeutic effects of platinum, a clinical trial using this agent in these patients is warranted.

We are now conducting an open label proof of concept, single arm, phase I dose escalation study of SGI-110 in patients with refractory GCTs (NCT02429466) (Fig. 4). Based on the clinical trial in ovarian cancer noted above, patients will be treated with subcutaneous SGI-110 injections at 30 mg/m$^2$ on a daily basis for days 1-5 followed by cisplatin 100 mg/m$^2$ on day 8 every 21 days. We seek to bring forward the new concept of epigenetic targeting in refractory GCTs and set the stage for interventions targeting the refractory GCT epigenome, as well as guide and impact the design of future clinical investigations in refractory GCTs.

CONCLUSION

The tactic of using DNMTIs for cancer treatment is no longer isolated to hematologic malignancies. Due to their unique germ cell origins, germ cell tumors are associated with distinct epigenetics that may be exploited to treat this malignancy. Micro RNAs, specifically miR-371a-3p, have been shown in preclinical and early clinical studies to be promising biomarkers that may increase the sensitivity and specificity to track disease. Preclinical data indicates that GCTs are hypersensitive to hypomethylating agents perhaps due to a high level of the germ cell marker DNMT3B and that refractory GCTs can be resensitized with these agents. Clinical trials in ovarian cancer have demonstrated safety of DNMTI and platinum combination therapy which is currently being further investigated in phase II trials. Clinical trials of DNMTIs in platinum refractory GCT are warranted. We are currently conducting a phase I trial using SGI-110 to re-sensitize platinum refractory GCT patients to combat this deadly disease.

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Fig. (1).
Proposed distinctive patterns of DNA methylation among testicular germ cell tumors, normal cells and somatic solid cancers.
SGI-110 is a second generation hypomethylating agent. SGI-110 is a dinucleotide of decitabine and deoxyguanosine. Decitabine is rapidly eliminated by cytosine deaminase, limiting drug exposure in vivo. SGI-110 increases the in vivo exposure of decitabine by protecting it from deamination.
Fig. (3).
Low dose, SGI-110 dramatically effects growth of testicular germ cell tumor xenografts. Representative result of a testicular cancer xenograft treated with 2.0 mg/kg SGI-110 or vehicle for 5 days per week for 2 weeks (Albany and Spinella, unpublished).
Fig. (4).
Outline of the ongoing phase I protocol, NCT02429466, for the treatment of refractory testicular germ cell cancer patients.