Immunomodulatory action of the DNA methyltransferase inhibitor SGI-110 in epithelial ovarian cancer cells and xenografts

Pragya Srivastava1,1, Benjamin E Paluch2,1, Junko Matsuzaki3, Smitha R James2, Golda Collamat-Lai2, Pietro Taverna4, Adam R Karpf5,*, and Elizabeth A Griffiths1,*

1Department of Medicine; Roswell Park Cancer Institute; Buffalo NY USA; 2Department of Pharmacology and Therapeutics; Roswell Park Cancer Institute; Buffalo NY USA; 3Center for Immunotherapy; Roswell Park Cancer Institute; Buffalo NY USA; 4Astex Pharmaceuticals Inc.; Dublin, CA USA; 5Eppley Institute; Fred & Pamela Buffett Cancer Center; University of Nebraska Medical Center; Omaha, NE USA

*y-Co-First Authors: These authors contributed equally to the presented work.

Keywords: cancer testis antigens, cancer germline genes, DNA methylation, DNA methyltransferase inhibitors, epithelial ovarian cancer, epigenetics, immune modulation, SGI-110

Abbreviations: AZA, Azacitidine (5-azacytidine); CTA, Cancer-testis antigen or cancer-germline antigen; CTAG1B, Cancer/testis antigen 1B; DAC, Decitabine (5-aza-2’-deoxycitidine); DNA, Deoxyribonucleic acid; DNMTi, DNA methyltransferase inhibitor; EOC, Epithelial ovarian cancer; HLA, Human leukocyte antigen; ICAM-1, Intracellular Adhesion Molecule 1; LINE-1, Long interspersed nuclear element-1; MAGE-A, Melanoma antigen family A; MHC, Major histocompatibility complex; NY-ESO-1, New york esophageal squamous cell carcinoma 1; RNA, Ribonucleic acid.

We aimed to determine the effect of SGI-110 on methylation and expression of the cancer testis antigens (CTAs) NY-ESO-1 and MAGE-A in epithelial ovarian cancer (EOC) cells in vitro and in vivo and to establish the impact of SGI-110 on expression of major histocompatibility (MHC) class I and Intracellular Adhesion Molecule 1 (ICAM-1) on EOC cells, and on recognition of EOC cells by NY-ESO-1-specific CD8+ T-cells. We also tested the impact of combined SGI-110 and NY-ESO-1-specific CD8+ T-cells on tumor growth and/or murine survival in a xenograft setting. EOC cells were treated with SGI-110 in vitro at various concentrations and as tumor xenografts with 3 distinct dose schedules. Effects on global methylation (using LINE-1), NY-ESO-1 and MAGE-A methylation, mRNA, and protein expression were determined and compared to controls. SGI-110 treated EOC cells were evaluated for expression of immune-modulatory genes using flow cytometry, and were co-cultured with NY-ESO-1 specific T-cell clones to determine immune recognition. In vivo administration of SGI-110 and CD8+ T-cells was performed to determine anti-tumor effects on EOC xenografts. SGI-110 treatment induced hypomethylation and CTA gene expression in a dose dependent manner both in vitro and in vivo, at levels generally superior to azacitidine or decitabine. SGI-110 enhanced the expression of MHC I and ICAM-1, and enhanced recognition of EOC cells by NY-ESO-1-specific CD8+ T-cells. Sequential SGI-110 and antigen-specific CD8+ cell treatment restricted EOC tumor growth and enhanced survival in a xenograft setting. SGI-110 is an effective hypomethylating agent and immune modulator, and thus, an attractive candidate for combination with CTA-directed vaccines in EOC.

Introduction

Expression of cancer testis antigen (CTA) genes in epithelial ovarian cancer (EOC) has been extensively characterized.1,3 Patients harboring EOCs that express CTA genes demonstrate humoral and cell mediated immune responses, which can be associated with prolonged survival.1,4-8 A number of vaccine studies targeting the CTA gene product NY-ESO-1 have been undertaken in EOC in order to enhance such anti-tumor immunity.9,12 Variable outcomes in these trials may result from the observation that in CTA-expressing cancers, gene expression is heterogeneous, with some regions of the tumor expressing the antigen robustly, while others do not.1,2 Furthermore, cancers may down-regulate NY-ESO-1 expression during the course of vaccine therapy.13

© Pragya Srivastava, Benjamin E Paluch, Junko Matsuzaki, Smitha R James, Golda Collamat-Lai, Pietro Taverna, Adam R Karpf, and Elizabeth A Griffiths

*Correspondence to: Adam R Karpf; Email: adam.karpf@unmc.edu.; Elizabeth A Griffiths; Email: elizabeth.griffiths@roswellpark.org

Submitted: 03/17/2014; Revised: 01/09/2015; Accepted: 01/28/2015
http://dx.doi.org/10.1080/15592294.2015.1017198

This is an Open Access article distributed under the terms of the Creative Commons Attribution-Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0/), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited. The moral rights of the named author(s) have been asserted.
Tumor-associated expression of CTA genes is often mediated by promoter hypomethylation, which is also influenced by the genome wide DNA methylation status. Further supporting a role for DNA methylation in CTA gene regulation in EOC cells is the observation that the DNMTi decitabine (DAC, 5-aza-2'-deoxycytidine) induces CTA gene expression. Importantly, DAC treatment not only leads to CTA gene expression, but also induced protein expression and functional presentation of CTA epitopes on the surface of cancer cells. Enhancement of CTA-directed immunogenicity by DAC likely also involves the induction of class I Human Leukocyte Antigens (HLA) and co-stimulatory molecules by this agent.

The above results have prompted considerable interest in the potential for clinical combination of CTA vaccine therapy with DNMTi. DAC enhances expression of the CTA genes NY-ESO-1 and MAGE-A3/A6 in vitro and in patients; however, the drug is given intravenously and it has a short half-life due to rapid inactivation by cytidine deaminase. The other FDA-approved DNMTi, 5-aza-2'-cytidine (AZA), can have similar effects on gene expression and DNA methylation; however the incorporation of this drug predominantly into RNA (≈85%) complicates its in vivo mechanism of action; like DAC, it has a short half-life. Although DAC and AZA have shown significant activity in patients with myeloid cancers, Phase I single agent studies in solid tumors were disappointing, likely due to the relatively slower growth rate of solid tumors and the short half-life of both drugs. To overcome these pharmacokinetic limitations, a rationally designed novel dinucleotide comprised of deoxy-guanosine complexed through a phosphodiester linker was synthesized. This compound, SGI-110, allows for DAC-like activity as DAC in inducing CTA genes by SGI-110 correlated with gene induction, we determined the mRNA and protein expression of NY-ESO-1 and MAGE-A. Both EOC cell lines demonstrated an increase in NY-ESO-1 and MAGE-A3/6 mRNA following SGI-110 treatment, and gene induction was greater than that observed with AZA or DAC (Fig. 1B). Both EOC cell lines also showed marked induction of NY-ESO-1 and MAGE-A protein (Fig. 1C). Notably, AZA was less potent at inducing CTA mRNA and protein expression, particularly in A2780 cells, although its effect on DNA methylation was similar. This could be due to the drug's off-target effects related to RNA incorporation.

SGI-110 treatment induces DNA hypomethylation and CTA mRNA and protein expression in EOC xenografts using a daily x 5 days treatment schedule

We treated OVCAR3 xenograft-bearing SCID mice with a series of clinically relevant dosing schedules of SGI-110 or DAC (schedules tested in the Phase I/II trial for MDS or AML, see Materials and Methods). We did not analyze AZA, as this drug was less potent in vitro in affecting CTA-related endpoints as compared to SGI-110 or DAC (Fig. 1). The effect of SGI-110 treatment on LINE-1, NY-ESO-1, and MAGE-A3/6 methylation was evaluated in excised OVCAR3 tumors as mentioned above. Groups 1 to 5 (see Table 1 for Group description) were exposed subcutaneously to SGI-110 at doses of 3, 6.1, or 10 mg/kg, or DAC at 2.5 mg/kg, daily for 5 days and tumors were harvested on day 7. Due to differences in molecular weight, the molar equivalent of a 1mg dose of DAC is approximately 2.5 mg of SGI -110, thus the 6.1mg dose of SGI-110, approximates the 2.5 mg/kg DAC dose. Mice treated on the 5 day schedule with SGI-110 at the 10 mg/kg/dose developed significant gastrointestinal toxicity. Both DAC and SGI-110 treatment caused hypomethylation of LINE-1 and NY-ESO-1 at all doses (Fig. 2A). MAGE-A3/6 hypomethylation was apparent at the 6.1 mg/kg SGI-110 dose (Fig. 2B). Tumors excised on day 7 demonstrated induction of NY-ESO-1 and MAGE-A3/6 mRNA with SGI-110 treatment (Fig. 2B). Robust induction of CTA protein expression following SGI-110 treatment was also observed, and appeared to be more robust than that seen with DAC treatment (Fig. 2C).
SGI-110 treatment induces DNA hypomethylation and CTA mRNA and protein expression in EOC xenografts using a weekly x 3 treatment schedule

Mice in treatment groups 6 to 10 received SGI-110 at 6.1, 12.2, or 24.4 mg/kg, or DAC at 5 mg/kg, once a week for 3 weeks. SGI-110 treatment resulted in significant LINE-1 and NY-ESO-1 hypomethylation on both days 7 and 16 post-treatment (Fig. 3A). SGI-110 at the highest dose (24.4 mg/kg) led to the greatest hypomethylation, without apparent toxicity (data not shown). Using MSP, we evaluated MAGE-A3/6 promoter methylation and observed hypomethylation on both days 7 and 16 (Fig. S2C). This dosing schedule induced mRNA and protein expression of NY-ESO-1 and MAGE-A3/6.

SGI-110 treatment induces DNA hypomethylation and CTA mRNA and protein expression in EOC xenografts using a weekly x 3 treatment schedule

Mice in treatment groups 6 to 10 received SGI-110 at 6.1, 12.2, or 24.4 mg/kg, or DAC at 5 mg/kg, once a week for 3 weeks. SGI-110 treatment resulted in significant LINE-1 and NY-ESO-1 hypomethylation on both days 7 and 16 post-treatment (Fig. 3A). SGI-110 at the highest dose (24.4 mg/kg) led to the greatest hypomethylation, without apparent toxicity (data not shown). Using MSP, we evaluated MAGE-A3/6 promoter methylation and observed hypomethylation on both days 7 and 16 (Fig. S2C). This dosing schedule induced mRNA and protein expression of NY-ESO-1 and MAGE-A3/6.

Table 1. Treatment schedule for xenograft experiments

| Groups   | Treatment mg/kg/d | Schedule                                                                 |
|----------|-------------------|--------------------------------------------------------------------------|
| G1-5     | Vehicle           | Treated daily for 5 days and tumor harvested on day 7.                    |
|          | SGI-110-3         |                                                                          |
|          | SGI-110-6.1       |                                                                          |
|          | SGI-110-10        |                                                                          |
|          | DAC-2.5           |                                                                          |
| G6-10    | Vehicle           | Treated once weekly for 3 weeks. One tumor was harvested on day 7 and the other on day 16. |
|          | SGI-110-6.1       |                                                                          |
|          | SGI-110-12.2      |                                                                          |
|          | SGI-110-24.4      |                                                                          |
|          | DAC-5             |                                                                          |
| G11-15   | Vehicle           | Treated twice weekly for 3 weeks. One tumor was harvested on day 7 and the other on day 16. |
|          | SGI-110-6.1       |                                                                          |
|          | SGI-110-12.2      |                                                                          |
|          | SGI-110-24.4      |                                                                          |
|          | DAC-2.5           |                                                                          |
expression of both CTA genes, particularly at the highest dose level (Fig. 3B–C).

**SGI-110 treatment induces DNA hypomethylation and CTA mRNA and protein expression in EOC xenografts using a twice weekly x 3 treatment schedule**

Mice in groups 11 to 15 received SGI-110 at doses of 6.1, 12.2, 24.4 mg/kg, or DAC at 2.5 mg/kg, twice per week for 3 weeks, and tumors were harvested on days 7 and 16. Mice treated at the highest SGI-110 dose, 24.4 mg/kg demonstrated significant morbidity by day 16 (data not shown). This schedule resulted in significant LINE-1 and NY-ESO-1 promoter methylation at all SGI-110 doses (Fig. 4A). Additionally, SGI-110 at the 12.2 (molar equivalent of 4.9 mg DAC) and 24.4 mg/kg (molar equivalent to 9.8 mg/kg DAC) doses resulted in significantly greater hypomethylation than the DAC treatment control (Fig. 4A). MAGE-A3/6 hypomethylation was also apparent using this schedule of SGI-110 (Fig. S2D). Again, hypomethylation of CTA promoters resulted in CTA gene induction at both the mRNA and protein levels (Fig. 4B–C). Specific SGI-110 treatment groups showed enhanced expression of CTA genes as compared to DAC. Unexpectedly, there was not a significant change in MAGE-A3/6 mRNA expression at day 7 (Fig. 4B); however, MAGE-A protein expression was induced with all DNMTi treatments, and expression was sustained between days 7 and 16. This likely reflects the fact that the western blot assay detects many homologous MAGEA family members, while primers for MAGE A3/6 are relatively specific.32

**SGI-110 treatment enhances cell surface expression of MHC class I and ICAM-1 in EOC cells**

To determine whether SGI-110 treatment enhanced the expression of MHC class I, HLA-A, B, C (HLA-ABC) and/or the costimulatory molecule ICAM-1 in EOC cells, we treated A2780 and OVCAR3 cells in vitro and measured pan HLA-ABC and ICAM-1 using flow cytometry. We observed SGI-110 concentration-dependent upregulation of HLA-ABC and ICAM-1 (Fig. 5A–B). In general, similar effects were observed following AZA and DAC treatment.

**SGI-110 treatment promotes NY-ESO-1-specific CD8+ T cell recognition of EOC cells**

To determine if SGI-110 mediated induction of NY-ESO-1 and immune-modulatory molecules results in enhanced immune
recognition, we co-cultured NY-ESO-1-specific CD8+ T-cells with OVCAR3 cells pretreated with DNMTi, and measured CD107a and CD107b expression, an established measure of antigen-specific CD8+ T cell-mediated cytolytic capacity. 33 SGI-110 treatment led to a significant increase in CD107a/b staining in NY-ESO-1 specific CD8+ T-cells as compared to vehicle control (Fig. 5C). A similar effect was observed with DAC treatment. These data indicate that SGI-110 treatment enhances NY-ESO-1 specific CD8+ T-cell recognition of HLA compatible tumor cells.

SGI-110 enhances NY-ESO-1-specific CD8+ T cell antitumor response in vivo

Finally, we sought to determine if the observed increase in CTA expression and other immune molecules translated into enhanced tumor control in vivo. For this task, SCID mice with established OVCAR3 xenografts were treated with SGI-110 and/ or NY-ESO-1-specific CD8+ T-cells. As 3.0 mg/kg SGI-110 daily x 5 induced NY-ESO-1 expression in vivo without significant toxicity, we chose this dose for subcutaneous treatment of mice. Three days later, mice were injected intratumorally with NY-ESO-1-specific, HLA compatible, CD8+ T-cells or vehicle control (PBS). The combination of SGI-110 and NY-ESO-1 specific T-cells showed delayed tumor growth in comparison with mice treated with SGI-110 or NY-ESO-1-specific CD8+ T-cells alone (Fig. 6A). This delayed tumor growth was associated with a statistically significant increase in survival for mice treated with the combination of SGI-110 and NY-ESO-1-specific CD8+ T-cells (Fig. 6B). In these experiments, mice were sacrificed once the tumor diameter reached 2 cm and therefore longer survival reflects slower tumor growth. Together, these data suggest SGI-110 treatment enhances NY-ESO-1-specific antitumor responses in vivo.

Discussion

We demonstrate herein that SGI-110 efficiently induces CTA promoter hypomethylation and gene expression in EOC cell lines in vitro. Furthermore we show that subcutaneous exposure of EOC tumor bearing mice to clinically relevant doses and schedules
of SGI-110 leads to CTA promoter hypomethylation, mRNA, and protein expression. SGI-110 treatment also increases expression of HLA-ABC and ICAM-1, likely further promoting in vitro recognition of EOC cells by antigen specific CD8+ T-cell clones. Tumor-bearing SCID mice treated with SGI-110 and subsequently intratumorally injected with HLA-compatible NY-ESO-1 specific T-cell clones showed delayed tumor growth compared with SGI-110 treatment or CD8+ T-cells alone. Taken together, these data suggest that SGI-110 enhances immune recognition of cancer cells both by inducing the expression of tumor antigens and by upregulating MHC class I and immunomodulatory molecules. Interestingly, SGI-110 appeared to be more potent than DAC at inducing expression of the CTAs MAGE-A and NY-ESO-1, particularly at the protein level. All 3 of the tested in vivo administration schedules of SGI-110 (daily x 5; weekly x 3; twice weekly x 3) were efficient at causing CTA promoter hypomethylation and inducing CTA gene expression. All 3 schedules have been investigated in the recently completed Phase I/II clinical trial for AML and MDS, and the daily x 5 schedule has been taken forward; in contrast the weekly x 3 schedule produced minimal methylation changes and was discontinued. In contrast, our current data support either of these schedules for potential combination with NY-ESO-1 vaccination in EOC patients. Prior reports supporting the notion that tumor specific T-cells and/or in combination with DAC can suppress tumor growth are consistent with our observations using SGI-110. Additionally, Fang et al. recently reported that SGI-110 reduces ovarian tumors growth both alone and in combination with cisplatin, and can act as a chemosensitizer, demonstrating activity in other contexts for this agent in EOC. Here we found that SGI-110 enhances NY-ESO-1 specific CD8+ T cell responses, and delayed tumor growth in a manor analogous to that reported in other contexts with DAC. Overall, these data have 2 major implications: i) induction of CTAs and related immunomodulatory activities should be considered as a potential mechanism of action of SGI-110 in ongoing trials of EOC and other solid tumors, and ii) SGI-110 should be considered as a potential agent to augment the efficacy of CTA-directed vaccines in trials of EOC and other relevant tumor types, including melanoma and lung cancer. The immunological effects of DNMTi are also supported by previous clinical studies of DAC. In this context, we have recently shown in a Phase I trial that DAC combination therapy with NY-ESO-1 vaccine and liposomal doxorubicin is

![Figure 4. SGI-110 treatment induces hypomethylation and expression of NY-ESO-1 and MAGE-A3/6 in subcutaneous OVCAR3 xenografts treated using a twice weekly x 3 week schedule. SGI-110 at varying doses (6.1, 12.2, 24.4 mg/kg) or DAC (2.5 mg/kg) was administered twice per week for 3 weeks, and tumors were harvested on days 7 and 16. (A) LINE-1 and NY-ESO-1 promoter methylation were determined by bisulfite pyrosequencing. (B) NY-ESO-1 and MAGE-A3/6 mRNA levels were determined by RT-qPCR. C) NY-ESO-1 and MAGE-A protein expression by western blotting. Data represent 3 animals/group. *P < 0.05 vs. vehicle; **P < 0.05 vs. DAC.](image-url)
safe, promotes immunological responses, and may provide clinical benefit in EOC patients.\(^{38}\) In addition to DNA methylation, CTA genes are regulated by other epigenetic mechanisms including histone acetylation, histone methylation, and nucleosome occupancy.\(^{41-43}\) Thus, agents that impinge on these processes may also be useful for combination with CTA-directed vaccination strategies. In summary, the current data suggest that SGI-110 is a promising agent to incorporate into the spectrum of novel epigenetic/immunological approaches for EOC.

![Figure 5. SGI-110 enhances HLA-ABC and ICAM-1 expression, and NY-ESO-1-specific CD8+ T cell immune recognition, in EOC cells treated in vitro. OVCAR3 and A2780 cells were treated with SGI-110, DAC, or AZA, and expression of (A) HLA-ABC and (B) ICAM-1 were determined by flow cytometry. Data represent the Log2 transformation of [median fluorescence intensity (MFI) of treatment / MFI of vehicle control]. (C) OVCAR3 cells were treated with SGI-110, DAC, or AZA at the concentrations shown. Following treatment, cells were cultured with NY-ESO-1 specific, HLA compatible CD8+ T cell clones derived from ovarian cancer patients and relative CD107a/b expression was determined. All experiments were repeated 3 independent times. *P < 0.05 vs. vehicle, NP-No peptide control.](image)

### Materials and Methods

**Cell lines and in vitro drug treatments**

OVCAR3 and A2780 EOC cell lines were propagated in DMEM supplemented with 10% Fetal Bovine Serum (FBS), 2 mM L, and 1% Penicillin-Streptomycin. SGI-110 was provided by Astex Pharmaceuticals, Inc. (Dublin, CA). AZA and DAC were obtained from Sigma. Cell lines were treated with SGI-110 at concentration 0.1, 1.0 and 5 μmol/L (in PBS) on
day 2 and day 4, and harvested on day 6. As controls, cells were treated with vehicle alone, 0.5 μmol/L DAC, and 2.0 μmol/L AZA using the same treatment schedule as SGI-110. Trypan blue exclusion was used to evaluate cell viability (Fig. S1).

Xenograft experiments
A total of 1 × 10^6 OVCAR3 cells suspended in Matrigel (BD Biosciences) were implanted into the hindquarters of 6 week old female SCID mice using an IACUC-approved protocol. After ~2–3 weeks, when macroscopic tumors were formed (~50 mm^3), mice were assigned into the different groups (Table 1). All treatment groups were exposed to vehicle, SGI-110 or DAC subcutaneously. For groups 1–5 a single tumor was implanted in one hindquarter and for groups 6–15 2 tumors were implanted, one in each hindquarter. Tumors were excised for molecular analysis on day 7 for Groups 1–5, and on days 7 and 16 for Groups 6–15.

DNA Methylation Analysis
Genomic DNA was isolated using the Puregene kit (Qia-gen) and sodium bisulfite conversion was performed using the EZ DNA Methylation Kit (Zymo Research). *NY-ESO-1* promoter and *LINE-1* repetitive element DNA methylation were determined by sodium bisulfite pyrosequencing as described previously. To evaluate *MAGE-A3/6* promoter methylation, MSP was performed as previously described. Primers were designed using MethPrimer. Primer sequences were used as previously reported. Unmethylated and methylated PCR products were amplified with initial denaturation at 95°C for 5 min, then 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 60 s, followed by a final 5 min extension at 72°C. PCR products were analyzed on 2% agarose gel by ethidium bromide staining.

Reverse Transcriptase quantitative PCR (RT-qPCR)
Total RNA was purified using Trizol (Invitrogen) and cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad). Absolute quantification was performed using PCR Master Mix for SYBR Green assays (Eurogentech) on 7300 Real-Time PCR System (Applied Biosciences). All samples were run in triplicate, and *NY-ESO-1* and *MAGE-A3/6* gene expression data were normalized to 18SrRNA. Primer sequences used were reported previously.

Western blot analysis
Total cellular protein was extracted from cell lines and/ or tissue samples using NP-40 lysis buffer and quantitated, and protein expression for *NY-ESO-1* and *MAGE-A* was evaluated as described previously. Figure S3 shows representative blots with molecular weight of the proteins studied along with molecular weight marker.

Flow cytometry
Cells were harvested and stained with anti-human HLA-ABC (clone W6/32) or anti-human CD54 (alternatively known as ICAM-1, clone HA58) antibodies (eBioscience) and changes in the expression of HLA-ABC and ICAM-1 following DNMTi treatment was determined as described previously. Representative gating is shown in Figure S4.

NY-ESO-1-specific CD8+ T cell recognition assay
NY-ESO-1-specific HLA-A2-restricted CD8+ T-cells were prepared as described previously, and HLA-A2 positive OVCAR3 cells were treated with AZA, DAC, or varying doses of SGI-110 as described above. Following treatment, cells were co-cultured for 6 hours at 37°C in the presence of anti-CD107a and CD107b antibodies (BD Biosciences), monensin, and brefeldin A (Sigma). Negative control stimulations with no peptide and positive control stimulations with peptide
(NY-ESO-157–165) or phorbol myristate acetate and ionomycin were analyzed in parallel. Using NY-ESO-1-specific CD8+ T-cells (identified as tetramer+ CD8+ cells), CD107a/b+ was determined and normalized to positive control which served as an internal control from each independent experiment.

NY-ESO-1-specific CD8+ T-cell antitumor response

OVCA3 cells (1 x 10⁶) suspended in PBS were subcutaneously implanted into the flanks of 5 week old female SCID mice using an IACUC-approved protocol. After ~2–3 weeks, when macroscopic tumors formed, mice were treated daily with either 3.0 mg/kg/d SGI-110 or vehicle control (diluent) and, 3 days later, intra-tumorally injected with 2 x 10⁶ NY-ESO-1-specific CD8+ T-cells or vehicle control (PBS). In total, there were 4 treatment groups (which included at least 7 mice/group): G1, vehicle (diluent and PBS); G2, NY-ESO-1-specific CD8+ T-cells (diluent and NY-ESO-1-specific CD8+ T-cells); G3, SGI-110 (SGI-110 and PBS) and G4, SGI-110 and NY-ESO-1-specific CD8+ T-cells. Tumors were measured according to the shortest (A) and longest diameter (B) to determine tumor volume (V) in which V = (A²B)/2. Survival was determined on the basis of tumor diameter. If a tumor reached 2cm in diameter, the mouse was euthanized.

Statistical analysis

Statistical analysis was performed by analysis of variance using GraphPad Prism. Differences between the 2 groups were analyzed using the t test. All data are presented as mean ± standard error. Difference between HLA-ABC and ICAM1 expression fold change was analyzed using one sample t test. The statistical significance of Kaplan-Meier survival curves was determined using log-rank (Mantel-Cox) test.

Disclosure of Conflicts of Interest

EAG and ARK received research funding from Astex Pharmaceuticals. EAG is a principal investigator on the Phase I/II clinical trial of SGI-110 sponsored by Astex Pharmaceuticals and has received honoraria from Celgene, Inc.. P.T. is an employee of Astex Pharmaceuticals. Other authors have no conflicts to disclose.

Acknowledgments

The authors would like to thank Drs. Michael Moser from the animal core facility at Roswell Park Cancer Institute and Dr. Michael Nemeth in the Department of Immunology at the Roswell Park Cancer Institute for helpful assistance.

Funding

BEP is supported by the Ruth L. Kirschstein National Research Service Award Institutional Training Grant (T32 CA009072). A.R.K. is supported by the Betty J. and Charles D. McKinsey Ovarian Cancer Research Fund and the Otis Gile Medical Research Foundation.

Supplemental Material

Supplemental data for this article is available on the publisher’s website.

References

1. Oudmii K, Jungbluth AA, Stockert E, Qian F, Gnjatic S, Tammela J, Intengan M, Beck A, Keitz B, Santiago D, et al. NY-ESO-1 and LAGE-1 cancer-testis antigens are potential targets for immunotherapy in epithelial ovarian cancer. Cancer Res 2003; 63:6067-83; PMID:14522938
2. Wroczynska-Read A, Mhawech-Fauceglia P, Yu J, Oudmii K, Karpf AR. Intertumoral and intratumoral NY-ESO-1 expression heterogeneity is associated with promoter-specific and global DNA methylation status in ovarian cancer. Clin Cancer Res 2008; 14:3283-90; PMID:18519754; http://dx.doi.org/10.1158/1078-0432.CCR-07-5279
3. Imura M, Yamashita S, Cai LY, Furuta J, Wakabayashi M, Yasugi T, Ushijima T. Methylation and expression analysis of 15 genes and three normally-methylated genes in 13 ovarian cancer cell lines. Cancer Lett 2006; 241:213-20; PMID:16303245; http://dx.doi.org/10.1016/j.canlet.2005.10.010
4. Gnjatic S, Atanackovic D, Jager E, Matsao M, Selvakumar M, Altorki NK, Maki RG, Dupont B, Ritter G, Chen YT, et al. Survey of naturally occurring CD4+ T cell responses against NY-ESO-1 in cancer patients: Correlation with antibody responses. Proc Natl Acad Sci U S A 2003; 100:8862-7; PMID:12853579; http://dx.doi.org/10.1073/pnas.1135324100
5. Stone B, Schummer M, Paley JP, Thompson L, Stewart J, Ford M, Crawford M, Urban N, O’Brian K, Nelson BH. Serological analysis of ovarian tumor antigens reveals a bias toward antigens encoded on 17q. Int J Cancer 2003; 104:75-84; PMID:12533422; http://dx.doi.org/10.1002/ijc.10900
6. Huarte E, Karbach J, Gnjatic S, Bender A, Jager D, Arand M, Atanackovic D, Skipper J, Ritter G, Chen YT, et al. HLA-DPγ4 expression and immunity to NY-ESO-1: Correlation and characterization of cytotoxic CD8+ CD25- CD8+ T cell clones. Cancer Immun 2004; 4:15; PMID:15600300
7. Qian F, Gnjatic S, Jager E, Santiago D, Jungbluth A, Grande G, Schneider S, Keitz B, Drocoll D, Ritter G, et al. Th1/Th2 CD4+ T-cell responses against NY-ESO-1 in a HLA-DPB1*0401/0402 patients with epithelial ovarian cancer. Cancer Immun 2004; 4:12; PMID:15521719
8. Sato E, Olson SH, Ahl J, Bundy B, Nishikawa H, Qian F, Jungbluth AA, Frosina D, Gnjatic S, Ambrosone C, et al. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. Proc Natl Acad Sci U S A 2005; 102:18538-43; PMID:16344661; http://dx.doi.org/10.1073/pnas.0509182102
9. Oudmii K, Qian F, Matsuji K, Mhawech-Fauceglia P, Andrews C, Hoffman EW, Fan L, Ritter G, Villetta J, Thomas B, et al. Vaccination with an NY-ESO-1 peptide of HLA class I/II specificities induces integrated humoral and T cell responses in ovarian cancer. Proc Natl Acad Sci U S A 2007; 104:12837-42; PMID:17652318; http://dx.doi.org/10.1073/pnas.0701021104
10. Karbach J, Gnjatic S, Bender A, Neumann A, Weidemann E, Yuan J, Ferrara CA, Hoffman E, Old LJ, Altorki NK, et al. Tumor-reactive CD8+ T-cell responses after vaccination with NY-ESO-1 peptide, CPG 7909 and montanide ISA-51: Association with survival. Int J Cancer 2010; 126:909-18; PMID:19728336; http://dx.doi.org/10.1002/ijc.24850
11. Oudmii K, Matsuzaki J, Karbach J, Neumann A, Mhawech-Fauceglia P, Miller A, Beck A, Morrison CD, Ritter G, Godoy H, et al. Efficacy of vaccination with recombinant vaccine and Poloxalol vectors expressing NY-ESO-1 antigen in ovarian cancer and melanoma patients. Proc Natl Acad Sci U S A 2012; 109:5797-802; PMID:22454399; http://dx.doi.org/10.1073/pnas.1117208109
12. Sabbatini P, Tsuji T, Ferran L, Ritter E, Sedrak C, Tuballes K, Jungbluth AA, Ritter G, Agahajanian C, Bell-McGuinn K, et al. Phase I trial of overlapping long peptides from a tumor self-antigen and poly-ICLC shows rapid induction of integrated immune response in ovarian cancer patients. Clin Cancer Res 2012; 18:6497-508; PMID:23032745; http://dx.doi.org/10.1158/1078-0432.CCR-12-2189
13. Nicholas L, Chen W, Davis ID, Jackson HM, Dimopoulos N, Barrow C, Browning J, Macgregor D, Williams D, Hopkins W, et al. Immunoeediting and persistence of antigen-specific immunity in patients who have previously been vaccinated with NY-ESO-1 protein formulated in ISCOMATRIX. Cancer Immunol Immunother 2011; 60:3625-37; PMID:21698545; http://dx.doi.org/10.1007/s00262-011-1041-3
14. De Smet C, Loriot A, Boon T. Promoter-dependent mechanism leading to selective hypomethylation within the 5′ region of gene MAGE-A1 in tumors. Mol Cell Biol 2004; 24:4781-90; PMID:15134172; http://dx.doi.org/10.1128/MCB.24.11.4781-4790.2004
15. Aker S, Odunsi K, Karpf AR. Regulation of cancer germline antigen gene expression: Implications for cancer immunotherapy. Future Oncol 2010; 6:717-32; PMID:20465387; http://dx.doi.org/10.2217/fon.10.36
16. Loriot A, De Plaen E, Boon T, De Smet C. Transient down-regulation of DNMT1 methyltransferase leads to activation and stable hypomethylation of...
MAGE-A1 in melanoma cells. J Biol Chem 2006; 281:10118-26; PMID:16497664; http://dx.doi.org/10.1074/jbc.M104692020 [pii]

Weber J, Salgaller M, Samid D, Johnson B, Herlyn M, Lassar N, Treisman J, Rosenberg SA. Expression of the MAGE-I tumor antigen is up-regulated by the demethylating agent 5-aza-2'-deoxycytidine. Cancer Res 1994; 54:1766-71; PMID:7511051

Karpf AR, Laak AW, Rinse TO, Hanke AN, Grossman D, Jones DA. Limited gene activation in tumor and normal epithelial cells treated with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine. Mol Pharmacol 2004; 65:18-27; PMID:14722233; http://dx.doi.org/10.1124/mol.65.1.18

Srivastava P, Palach BE, Matuzaki J, James SR, Collamat-Lai G, Karbach J, Nemeth M, Taverna P, Karpf AR, Griffiths EA. Immunomodulatory action of SGI-110, a 5-aza-2'-deoxycytidine. Mol Immunol 2009; 58:589-601; PMID:18791715; http://dx.doi.org/10.1016/j.molimm.2009.04.001

Adair SJ, Hogan KT. Treatment of ovarian cancer cell lines with 5-aza-2'-deoxycytidine upregulates the expression of cancer-testis antigens and class I major histocompatibility complex-encoded molecules. Cancer Immunol Immunother 2009; 58:589-601; PMID:18791715; http://dx.doi.org/10.1007/s00262-008-0582-6

Petersi MI, Giaccone G, Colzi F, Danielli R, Fratta E, Covre A, Taverna P, Sigalotti L, Maiò M. Immunomodulatory activity of SGI-110, a 5-aza-2'-deoxycytidine-containing demethylating dinucleotide. Cancer Immunol Immunother 2013; 62:605-14; PMID:23941873; http://dx.doi.org/10.1007/s00262-012-1365-7

Karpf AR. A potential role for epigenetic modulatory drugs in the enhancement of cancer/germ-line antigen vaccine efficacy. Epigenetics 2006; 1:116-20; PMID:17124784

Griffiths EA, Gore SD. Epigenetic therapies in MDS and AML. Adv Exp Med Biol 2013; 754:253-83; PMID:24194994; http://dx.doi.org/10.1007/978-1-078-0432.CCR-10-2135

Kantarjian H, Roboz G, Rizziere D, Stock W, O’Connell G, Griffiths EA, Yee K, Tches B, Garcia-Manero G, Ravandi F, et al. Results from the dose escalation phase of a randomized phase 1 dose in cancer patients with relapsed/refractory MDS and AML. Blood 2012; 120:414-18

Bellet RE, Catalano RB, Mastrangelo MJ, Berd D. Phase II study of subcutaneously administered 5-azacytidine (NSC-108216) in patients with metastatic malignant melanoma. Med Pediatr Oncol 1978; 4:111-5; PMID:681640; http://dx.doi.org/10.1007/s00262-006-0582-6

Yoo CB, Jeong S, Egger G, Liang G, Phiasivongsa P, Tang C, Redkar S, Jones DA. Delivery of 5-aza-2'-deoxycytidine to cells using oligodeoxynucleotides. Cancer Res 2007; 67:6400-8; PMID:17616700; http://dx.doi.org/10.1158/0008-5472.CAN-06-3898

Griffiths EA, Choy G, Redkar S, Taverna P, Azab M, Karpf AR, SGI-110. DNA methyltransferase inhibitor, oncologic. Drugs Fut 2013; 38:355.

Stresemann C, Lyko F. Modes of action of the DNA methyltransferase inhibitors azacitadine and decitabine. Int J Cancer 2008; 128:8-13; PMID:18425818; http://dx.doi.org/10.1002/ijc.25607

Woloszyńska-Read A, Zhang W, Yu J, Link PA, Mhawech-Fauceglia P, Collamat G, Akers SN, Ostler KR, Godley LA, Odunsi K, et al. Coordination cancer germline antigen promoter and global DNA hypomethylation in ovarian cancer: Association with the BORIS/CTCF expression ratio and advanced stage. Clin Cancer Res 2011; 17:2170-80; PMID:21296871; http://dx.doi.org/10.1158/1078-0432.CCR-10-2135

Kantarjian H, Roboz G, Rizziere D, Stock W, O’Connell G, Griffiths EA, Yee K, Tches B, Garcia-Manero G, Ravandi F, et al. Results from the dose escalation phase of a randomized phase 1 dose in cancer patients with relapsed/refractory MDS and AML. Blood 2012; 120:414-18

Meliotti A, et al. Epigenetic potentiation of NY-ESO-1 vaccine therapy in human ovarian cancer. Cancer Immunol Res 2014; 2:37-49

Matsi D, Fang S, Shen C, Schilder J, Arnold A, Zeng Y, Berry WA, Huang T, Nephew KP. Epigenetic reselement to platinum in ovarian cancer. Cancer Res 2012; 72:2197-205; PMID:22549947; http://dx.doi.org/10.1158/0008-5472.CAN-11-3909

Scheppe DS, Fischette MR, Nguyen DM, Zhao M, Li X, Kunst TF, Hancos A, Hong JA, Chen GA, Pischke V, et al. Phase 1 study of decitabine-mediated gene expression in patients with cancers involving the lungs, esophagus, or pleura. Clin Cancer Res 2006; 12:5777-85; PMID:17090214; http://dx.doi.org/10.1158/1078-0432.CCR-06-0669

Link PA, Gangisetty O, James SR, Woloszyńska-Read A, Tachibana M, Shinaki Y, Karpf AR. Distinct roles for histone methyltransferases G9a and GL in cancer gene repression in human cancer cells and murine embryonic stem cells. Mol Cancer Res 2009; 7:851-62; PMID:19531572; http://dx.doi.org/10.1158/1545-7186.MCR-08-0497

Wischnewski F, Pankei K, Schwarzewich H. Promoter demethylation of iCAD, a tumor suppressor gene, in colorectal cancer cells. Clin Cancer Res 2008; 14:4324-32; PMID:18515385; http://dx.doi.org/10.1158/1078-0432.CCR-08-0653

James SR, Cederon CD, Sharma A, Zhang W, Mohler JF, Odunsi K, Wilson EM, Karpf AR. DNA methylation and nucleosome occupancy regulate the cancer germline antigen gene MAGEA11. Epigenetics 2013; 8:849-63; PMID:23893253; http://dx.doi.org/10.4161/epi.25500

Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A 1996; 93:9821-6; PMID:8790415

Li LC, Dahya R. MethPrimer: Designing primers for methylation PCR. Bioinformatics 2002; 18:1427-31; PMID:12424112