

Original Article

Differences in MEK inhibitor efficacy in molecularly characterized low-grade serous ovarian cancer cell lines

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Abstract: Advanced or recurrent low-grade serous ovarian cancers (LGSC) are resistant to conventional systemic treatments. LGSC carry mutations in *RAS* or *RAF*, leading to several clinical trials evaluating MEK inhibitors (MEKi). As LGSC cell lines and xenografts have been difficult to establish, little is known about the efficacy and on-target activity of MEKi treatment in this disease. We compared four different MEKi (trametinib, selumetinib, binimetinib and refametinib) in novel LGSC patient-derived cell lines. Molecular characterization of these cells included copy-number variation and hotspot mutational analysis. Proliferation, apoptosis and cell viability assays were used to study drug efficacy. MEKi on-target efficacy was measured using western blotting and isoelectric point focusing for ERK1/2 phosphorylation. Ten LGSC cell lines were derived from 7 patients with advanced/recurrent disease. Copy number variation showed significant heterogeneity among cell lines, however all samples showed deletions in chromosome 9p21.3, and frequent copy number gains in chromosomes 12 and 20. Mutations in *KRAS/NRAS* were identified in 4 patients (57%) and *RAS* mutation status was not associated with higher baseline levels of ERK phosphorylation. Different degrees of MEKi sensitivity were observed in the LGSC cell lines. Two cell lines, both with *KRAS* mutations, were highly sensitive to MEKi. Drug anti-proliferative efficacy correlated with the degree of inhibition of ERK phosphorylation, with trametinib being the most potent agent. Differences in MEKi efficacy were observed in LGSC cell lines. Trametinib showed the greatest anti-proliferative effects. This study serves as a basis for much needed future research on MEKi drug efficacy in LGSC.

Keywords: Low-grade serous ovarian cancer, cell line models, gene mutations, copy-number alterations, MAPK signalling, MEK inhibitors, ERK1/2 phosphorylation

Introduction

LGSC is much less common than its high-grade serous counterpart (HGSC), however the 10-year survivals are similar for advanced/recurrent cases [1, 2]. LGSC is often diagnosed in pre-menopausal women and the malignancy has distinct clinical, pathological and molecular features compared to HGSC [3-5]. As ovarian cancer is a heterogeneous disease, it is important that the various molecular subtypes are studied as distinct diseases [6]. In cases of

advanced and/or recurrent disease, tumor growth and progression is more indolent than HGSC, however the disease usually proves to be fatal [2]. LGSC responds poorly to anti-hormone therapy (9%) or chemotherapy, with response rates of less than 5% with chemotherapy treatment in the neoadjuvant or relapsed setting [7-12].

The clinical precursors that lead to the development of LGSC have been characterized. Serous ovarian borderline tumors (SBOT) are known

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precursor lesions. Certain pathological features associated with SBOT, such as the micropapillary variant (MPSBT), or the presence of invasive implants are risk factors for developing LGSC [13, 14]. Alternatively, some cases arise as primary LGSC presenting with either localized or metastatic disease. Molecular characterization of SBOTs, MPSBT and LGSC [15, 16] has established a linkage between LGSC and its precursors, resulting in the pathological classification of LGSC as a distinct entity. Whole exome sequencing of LGSC has been reported and shown that these tumors contain few point mutations when compared to HGSC [17].

The Gynecologic Cancer InterGroup (GCIg) Consensus Conference recently affirmed an urgent need to develop specific treatment options for patients with advanced/recurrent LGSC [18, 19]. Investigational studies using MEKi in LGSC are ongoing and previously predicated on experimental evidence showing activated MAPK signalling as a consequence of either *BRAF* or *RAS* mutations, often found in LGSC [20]. Activating mutations in *RAS* genes are one of the most common mutations found in cancer and multiple allosteric MEK inhibitors (MEKi), have been developed and used in many cancer types [21, 22]. Recently, an important phase II clinical trial evaluated the MEKi selumetinib, reporting response rates of 15% in patients with recurrent LGSC [23]. These results compare favourably with a previous cohort study that reported response rates of 4% using conventional chemotherapy [12]. Several MEKi are now being tested in clinical trials in patients with advanced/recurrent LGSC [NCT00551070, NCT01849874, NCT02101788]. Remarkably, preclinical testing of MEKi in LGSC has been limited due to the absence of representative cell line and xenograft models [24]. Thus, the aim of our study was to establish and molecularly characterize novel LGSC cell lines derived from patients with advanced/recurrent disease in order to compare the efficacy of four different MEKi agents (trametinib, selumetinib, binimetinib and refametinib).

Materials and methods

Patients, tumor samples and clinical information

Advanced or recurrent LGSC samples (tumor and ascites) were obtained from tumor banks

(Vancouver General Hospital and the British Columbia Cancer Agency (BCCA); London Regional Cancer Program, London, Ontario, Canada). Tumor bank protocols, cell line derivation, and the research relating to this study, was conducted according to institutional human ethics review board approvals at the BCCA and the University of British Columbia (H14-02859 and R05-0119), and Western University (HSREB 12668E). Clinical information was extracted retrospectively from patient medical records. Tumor bank pathology was reported by certified gynecological pathologists to ensure diagnostic accuracy.

Establishment and maintenance of patient-derived LGSC cell lines

LGSC patient-derived cell lines were established in-house through continuous *in vitro* culture of primary patient material (tumor tissue or ascites) obtained through the OvCaRe Tumor bank or the London Translational Ovarian Cancer Research Program (iOvCa241 and iOvCa250). LGSC cells were established and maintained in M199:MCDB105 (1:1) media (Cat. No. M5017, Cat. No. M6395, Sigma-Aldrich, Oakville, Ontario, Canada) supplemented with 10% fetal bovine serum (dFBS; Cat. No. SH30070.03, Hyclone, GE Life Sciences, Logan, UT, USA) at 37°C and 5% CO₂. No immortalization methods were used.

Cell line authentication

Microsatellite Analysis of Short Tandem Repeats (STRs) was performed for cell line authentication. STR analyses of 10 markers/loci were performed by Genewiz Inc. (South Plainfield, NJ) ([Supplementary Table 1](#)).

Mutation testing and copy number analysis

DNA was extracted from all cell lines using All Prep DNA/RNA Mini kit (Cat. No. 80204, Qiagen, Toronto, ON, Canada) according to protocol instructions, and quantified using a NanoDrop 2000™ UV-Vis instrument (ThermoScientific, Burlington, ON, Canada). Sequencing libraries were created from cell DNA for molecular characterization using Ion Torrent AmpliSeq™ Cancer Hotspot Panel Version 2 (Life Technologies, Grand Island, NY, USA) as per manufacturer's protocols. A total of 50

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Table 1. Clinical information LGSC patients and samples

Patient Number	Age at Diagnosis	Primary Pathology	Disease Stage	Sample Number	Cell Line	Pathology at Collection	Treatment Status	Sample Origin	Mutation Status
Patient #1	58	SBOT with non-invasive implants	IIB	1	VOA-1312	LGSC	Naïve	Ascites	KRAS
Patient #2	51	SBOT with LGSC	IIIA	2	iOvCa241	LGSC	Post-chemotherapy	Ascites	KRAS
Patient #3	53	SBOT with LGSC	IIC	3	VOA-3667*	LGSC	Naïve	Tumor Tissue	KRAS, KDR
Patient #4	62	Micropapillary SBOT with invasive implants	IIIC	4	VOA-1056	LGSC	Naïve	Tumor Tissue	NRAS, FGFR3, JAK3
				5	VOA-3993	Recurrent LGSC	Post-chemotherapy	Tumor Tissue	NRAS, FGFR3, JAK3
Patient #5	42	Micropapillary SBOT	IC	6	VOA-3448	Recurrent LGSC	Post-chemotherapy	Tumor Tissue	KDR, MET, PIK3CA
				7	VOA-3723	Recurrent LGSC	Post-chemotherapy	Tumor Tissue	KDR, MET, PIK3CA
Patient #6	42	LGSC	IIIC	8	VOA-4627	Recurrent LGSC	Post-chemotherapy	Ascites	KIT, PIK3CA, TP53
				9	VOA-4698	Recurrent LGSC	Post-chemotherapy	Ascites	KIT, PIK3CA, TP53
Patient #7	61	Primary Peritoneal LGSC	IIB	10	iOvCa250	LGSC	Naïve	Ascites	APC, KDR, KIT

Note: SBOT = Serous Borderline Ovarian Tumor. (*) = Primary cell culture.

Table 2. Missense mutations identified in LGSC cell lines using Ion AmpliSeq Cancer Hotspot Panel v2 Analysis

Gene Name	Mutation ID	PATIENT 1		PATIENT 2		PATIENT 3		PATIENT 4		PATIENT 5		PATIENT 6		PATIENT 7	
		VOA-1312	iOvCa241	VOA-3667#	VOA-1056	VOA-3993	VOA-3448	VOA-3723	VOA-4627	VOA-4698	iOvCa250				
APC (Chr.5)	COSM19099														c.3949G>C (Hm)
FGFR3 (Chr.4)	COSM1539830				c.1156T>C (Ht)	c.1156T>C (Ht)									
JAK3* (Chr.19)	COSM34213				c.2164G>A (Ht)	c.2164G>A (Ht)									
KDR (Chr.4)	COSM149673			c.1416A>T (Ht)			c.1416A>T (Ht)	c.1416A>T (Ht)							c.1416A>T (Ht)
KIT (Chr.4)	COSM28026											c.1621A>C (Ht)	c.1621A>C (Ht)		c.1621A>C (Ht)
KRAS* (Chr.12)	COSM520, COSM521	c.35G>T (Ht)	c.35G>A (Ht)	c.35G>T (Ht)											
MET (Chr.7)	COSM5020653						c.1124A>G (Ht)	c.1124A>G (Ht)							
NRAS* (Chr.1)	COSM584				c.182A>G (Ht)	c.182A>G (Ht)									
PIK3CA (Chr.3)	COSM328028						c.1173A>G (Ht)	c.1173A>G (Ht)	c.1173A>G (Ht)	c.1173A>G (Ht)					
TP53* (Chr.17)	COSM99729								c.701G>A (Ht)	c.701G>A (Ht)					

Note: (*) Means missense mutations reported as oncogenic, and (#) transient primary cell culture. A total of 207 amplicons covering approximately 2,800 COSMIC mutations from 50 oncogenes and tumor suppressor genes: ABL1, EGFR, GNAS, KRAS, PTPN11, AKT1, ERBB2, GNAQ, MET, RB1, ALK, ERBB4, HNF1A, MLH1, RET, APC, EZH2, HRAS, MPL, SMAD4, ATM, FBXW7, IDH1, NOTCH1, SMARCB1, BRAF, FGFR1, JAK2, NPM1, SMO, CDH1, FGFR2, JAK3, NRAS, SRC, CDKN2A, FGFR3, IDH2, PDGFRA, STK11, CSF1R, FLT3, KDR, PIK3CA, TP53, CTNNB1, GNA11, KIT, PTEN, VHL.

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common oncogenes and tumor suppressor genes were screened (**Table 2**). Sanger sequencing was performed to confirm missense mutations using methods previously described [25]. Primer sequences used for verification are listed in [Supplementary Table 2](#). Priming sites for -12 M13 forward and -27 M13 reverse were added to the 5' ends to allow direct Sanger sequencing of amplicons [26]. CNV analysis was done using Illumina® HumanOmni 2.5M-8 Array or CytoScan® HD array (Affymetrix, Inc) according to manufacturer's protocols. Nexus Copy Number™ (BioDiscovery, Inc.) software was used to analyze the copy number data from these two platforms.

In vitro drug sensitivity assay

Trametinib (GSK1120212; Sellekchem, Cat. No. S2673), selumetinib (AZD6244; Cat. No. S1008), binimetinib (MEK162; Cat. No. S7007), and refametinib (Bay 86-9766; Cat. No. S1089) were purchased from Selleck Chemicals (Houston, TX, USA). Dimethylsulfoxide or DMSO (Sigma, Cat. No. D2650) was purchased from Sigma-Aldrich (Oakville, Ontario, Canada). LGSC cells were seeded in 96-well plates at 30-40% confluence. At 24 hours cells were treated with DMSO (control) or MEKi (trametinib, selumetinib, binimetinib, or refametinib) using a range of different drug concentrations. Inhibitory concentration (IC₅₀, or concentration for 50% of maximal inhibition of cell proliferation) was determined using crystal violet assay after 72 h drug treatment.

Immunoassays to detect ERK1/2 phosphorylation

Western blot analysis: LGSC cell lysates were prepared according to previously published protocols [27, 28]. Cell lysates (20 µg) were separated on 8% SDS-PAGE gel and transferred to nitrocellulose membranes. Primary antibodies included ERK1/2 (Millipore, Cat. No. 06-182), p-MAPK (p-ERK1/2, Cell signaling, Cat.No. 4376S), MEK1/2 (Santa Cruz Biotechnologies, Cat. No. 436), p-MEK1/2 (Cell Signaling, Cat. No. 9154S), c-PARP (Cell Signaling, Cat. No. 9541S). Vinculin (V9131, Sigma) was used as a protein loading control. The appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (goat-anti-mouse or goat-anti-rabbit, Sigma Cat. No. A9917 and A0545) were used accordingly. Western blots were imaged using Immobilon HRP reagent (Cat. No. WBKLS0500,

Millipore, Etobicoke, ON, Canada) and developed by autoradiograph.

Capillary isoelectric point focusing proteomic analysis: Native capillary isoelectric point focusing (cIEF) was performed to assess total and phosphorylated ERK isoform expression using NanoPro1000™ System (ProteinSimple™, Santa Clara, CA) according to manufacturer protocols [23]. G2 premix gradient (pH 5-8) (Cat. No. 040-972, ProteinSimple™), ERK 1/2 (Cat. No. 040-474, ProteinSimple™) primary antibody and Goat anti-Rabbit (Santa Cruz sc-2054) Human Absorbed IgG secondary antibody was used to identify the protein isoforms. For both cIEF and Western blot studies, cells were treated with 20 ng/ml of EGF (Cat. No. PHG0315, Invitrogen) for 10 minutes.

Cell proliferation assays

Cell proliferation was measured using the IncuCyte™ (Essen Biosciences, Ann Arbor, MI, USA). LGSC cell lines were plated at 15-20% confluence on 96 well plates. After 24 hours, cells were treated once with DMSO (control) or MEKi (trametinib 0.1 and 0.5 µM; selumetinib, binimetinib, and refametinib 1 and 5 µM). The IncuCyte, a real-time imaging system using a non-labeled cell monolayer confluence approach, was used to monitor the cell growth by taking phase contrast images of cells at six hour intervals for 5-6 days. Data analysis was performed using IncuCyte™ cell proliferation assay software. Results are representative of three independent experiments, containing five technical replicates.

Cell viability assay

Cell viability was measured using the MTS-Cell Titer 96R Aqueous Non-Radioactive Cell Proliferation Assay following the manufacturer's recommendations (Cat. No. G5430, Promega, Madison, WI, USA). LGSC cells were seeded at 15-20% confluence in 96-well plates. Cells were treated the next day with DMSO control or drug (trametinib -0.1 and 0.5 µM, or selumetinib, binimetinib, and refametinib at 1 and 5 µM) and incubated for 5-6 days. Then, the media in each well was replaced with 100 µL of fresh media and 20 µL of MTS reagent. Plates were incubated for 3.5 hours at 37°C in humidified 5% CO₂. Absorbance at 490 nm was measured using microplate reader (BioTek Epoch SN257811). Cell viability after MEKi treatment was compared to DMSO treated cells.

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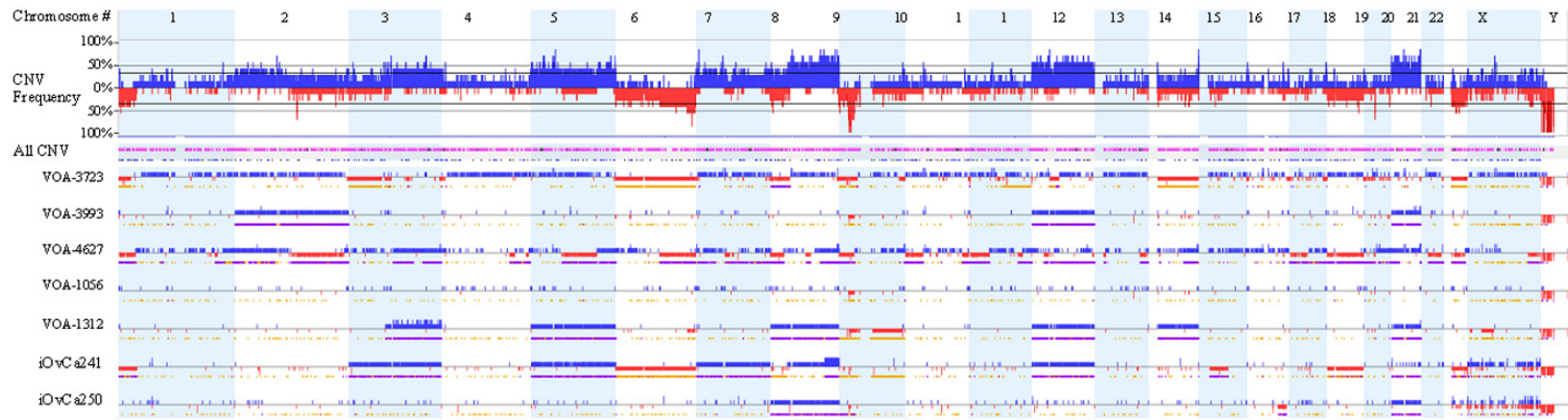


Figure 1. Graphical representation of copy number variation according to chromosome number. The top graph is a compilation of all cell lines with increased copy number in blue and decreased in red. For each individual cell line, regions of loss of heterozygosity are represented by a yellow line (below the copy number changes), whereas a purple line represents regions of allelic imbalance.

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Apoptosis assay

Apoptosis was measured using a Caspase-Glo® 3/7 Assay Kit (Cat. No. G8090, Promega, Madison, WI, USA). Briefly, iOvCa241, VOA-1056 and VOA-4627 LGSC cells (8×10^3 , 5×10^3 , 4×10^3 cells/96-well for 24 h treatment and, 6×10^3 , 4×10^3 , 3×10^3 cells/96-well for 72 h treatment) were incubated for 24 and 72 hours with vehicle (DMSO) or MEKi (trametinib 0.1 μ M and 0.5 μ M; or selumetinib, binimetinib or refametinib 1 μ M and 5 μ M). At the end of each treatment, luminescence of each well was measured in a plate-reading luminometer (Tecan Infinite M200Pro). Experiments were performed in triplicate for all conditions and cell lines.

Results

LGSC cell lines have unique cellular and molecular characteristics

Patient clinical data and cell line information used in this study are summarized in **Table 1**. All cell lines were derived from tumor tissues or ascites from patients with invasive LGSC (advanced or recurrent), though the original patient diagnosis varied as expected (borderline, micropapillary borderline and LGSC). A total of 10 LGSC cell cultures, derived from 7 different patients, were studied and molecularly characterized, though one became senescent (VOA-3667) and another (iOvCa250) grew too slowly to be used for most experiments. Three cell line pairs were derived from the same patient at different time-points in their disease (VOA 1056;VOA-3993, VOA-3448;VOA-3723, and VOA-4627;VOA-4698). Compared to other ovarian cancer cell lines, LGSC cell lines ([Supplementary Figure 1](#)) show vacuolization, strong cell adhesion properties (resistance to trypsinization), and low proliferation rates. STR analyses were used to define microsatellite loci for each new cell line and results were compared to the DSMZ STR profile website database (<https://www.dsmz.de/services/services-human-and-animal-cell-lines/online-str-analysis.html>), which confirmed the uniqueness of these cell lines.

Hotspot analysis in our LGSC cells revealed missense mutations in 10 oncogenes: *KDR*

(4/10 cell lines; 3 patients), *PIK3CA* (4/10; 2), *KRAS* (3/10; 3), *KIT* (3/10; 2), *NRAS* (2/10, 1), *MET* (2/10; 1), *FGFR3* (2/10; 1), *JAK3* (2/10; 1), *TP53* (2/10, 1), and *APC* (1/10; 1). As confirmed by Sanger sequencing, 1 to 3 missense mutations were detected per case (**Table 2**). Only 3 of these missense mutations (*KRAS*, *NRAS*, and *TP53* genes) are known to be pathogenic variants according to COSMIC and NCBI ClinVar classification (**Table 2**). Mutations in *RAS* (*KRAS*, *NRAS*) genes were the most frequent and they were detected in 5 out of 10 cell lines derived from 4 different patients (**Table 1**). No mutations in *BRAF* were found in the 10 cell lines, and *KRAS* and *NRAS* mutations were found to be mutually exclusive. Two cell lines (VOA-4627, VOA-4698), which were derived from the same patient, carried a *TP53* missense mutation. This uncommon finding has been confirmed histologically and recently reported as an unusual LGSC of the peritoneum, potentially arising from a low-grade serous tubal intra-epithelial carcinoma (STIC) in a patient harbouring a *BRCA1* mutation [29]. All missense mutations identified in the paired samples tested were stable over time.

Subsequent analysis of gene copy-number variation (CNV) was performed in 7 of the cell lines (derived from 6 patients) as shown in **Figure 1**. There were remarkable differences in the percentage of genomic aberrations (2-40%) among them, but interestingly all cell lines shared copy number loss affecting the chromosome region 9p21.3. This loss was homozygous in all but one cell line (VOA-4627). Amplification of chromosomes 8, 12 (containing the *KRAS* locus) and 20 were also commonly detected. Copy number gains in chromosomes 12 and 20 were detected in 5 out of 7 cell lines. Of note, chromosomes 4 and 13 were very well conserved. CNV changes were analyzed over time in one of our paired sample (VOA-1056 and VOA-3993). In this case, both cell lines shared similar copy number alteration profiles, however there were significant copy number gains in chromosomes 2, 12, and 20 in the recurrent sample (VOA-3993) following treatment with chemotherapy ([Supplementary Figure 2](#)). VOA-4627 showed loss of chromosome 1p, a finding that has been previously observed in LGSC [30] and also showed high levels of chromosomal copy number abnormalities and deletions than the other cell lines (**Figure 1**). Interestingly, all LGSC cell lines showed copy number changes

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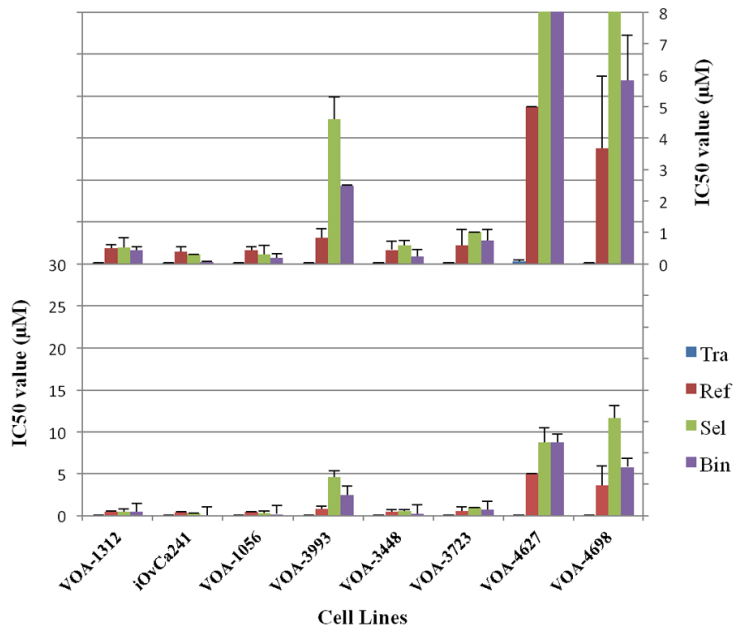


Figure 2. Inhibitory Concentration (IC₅₀) values of LGSC cell lines in response to different MEKi treatments. Tra = trametinib, Sel = selumetinib, Bin = binimetinib, Ref = refametinib. The top graph is an expanded scale of the bottom graph in order to better show the lower IC₅₀ values. Four replicates were performed for each treatment condition. All experiments were performed in triplicate.

(either gain or loss) in common MAPK-pathway related genes (Supplementary Table 3).

LGSC cell lines that carried oncogenic mutations in *KRAS* (iOvCa241, VOA-1312) and *NRAS* (VOA-1056, VOA-3993) genes were found to have copy number gain with allelic imbalance in the same chromosomal regions containing these loci (chr12:25,357,722-25,403,865 for *KRAS*, and chr1:115,247,084-115,259,515 for *NRAS*). Furthermore, iOvCa241 showed copy number gain in *BRAF*, and VOA-3993 showed copy number gain in *KRAS*. In the case of the unusual cell line carrying mutations in *TP53* and *BRCA1*, allelic imbalance in multiple regions without clear copy number change was identified. Copy number changes in relation to the genes tested for hot spot analysis, as shown in Supplementary Table 3.

LGSC cell lines have different sensitivities to MEK inhibitors

Drug sensitivity experiments were conducted using four different MEKi (trametinib, selumetinib, binimetinib and refametinib), the first three of which are investigational drugs for the treatment of advanced/recurrent LGSC patients. We determined the IC₅₀ of each drug in each LGSC cell line (Figure 2). Figure 2 shows a

comparison of the IC₅₀ for each drug, and all of the cell lines tested were most sensitive to trametinib with IC₅₀ values in the nM range (versus µM for all other drugs). Next, the same cell lines were used to study the impact of all four MEKi's on proliferation. Using a live cell-imaging instrument (Incucyte®), we assessed the effect of a single drug dose on cell proliferation over a 5 day period. Based on our IC₅₀ data and drug dosing from previous studies [31-33], we chose the following MEKi doses for these experiments: 0.1 µM trametinib, and 1 µM selumetinib/binimetinib/refametinib. MEKi treatments showed the greatest inhibitory effects on cell lines harbouring oncogenic mutations in *KRAS* (iOvCa241, VOA-1312). In keeping with the IC₅₀ results, trametinib showed the strongest inhibitory effect across all LGSC cell lines (Figure 3).

Notable differences in proliferation were observed between cell lines in response to MEKi treatment (Figure 3). Complete inhibition of proliferation was observed in iOvCa241 (*KRAS* G12D) and VOA-1312 (*KRAS* G12V) in response to treatment with any MEKi, and most of the cells remaining on the plate were visibly non-viable when the images were examined. Therefore, at the doses tested, drug efficacy was comparable for all MEKi in these two highly sensitive cell lines. In contrast, MEKi treatment in the other cell lines showed less anti-proliferative effects and the observed effects depended on the specific MEKi used for treatment. In the less sensitive cells (VOA-1056, VOA-3993, VOA-3448, VOA-3723, VOA-4627, VOA-4698), trametinib showed the greatest anti-proliferative activity (Figure 3, patients #3, #4, #5). Using an alternative assay to measure cell viability (tetrazolium assay reagent; data not shown) we confirmed the differential cell sensitivity and MEKi efficacy results detected in the proliferation assays (data not shown).

MEK inhibitors vary in their efficacy at blocking ERK1/2 phosphorylation

After detecting different proliferation and viability effects among the four MEKi's, we analyzed

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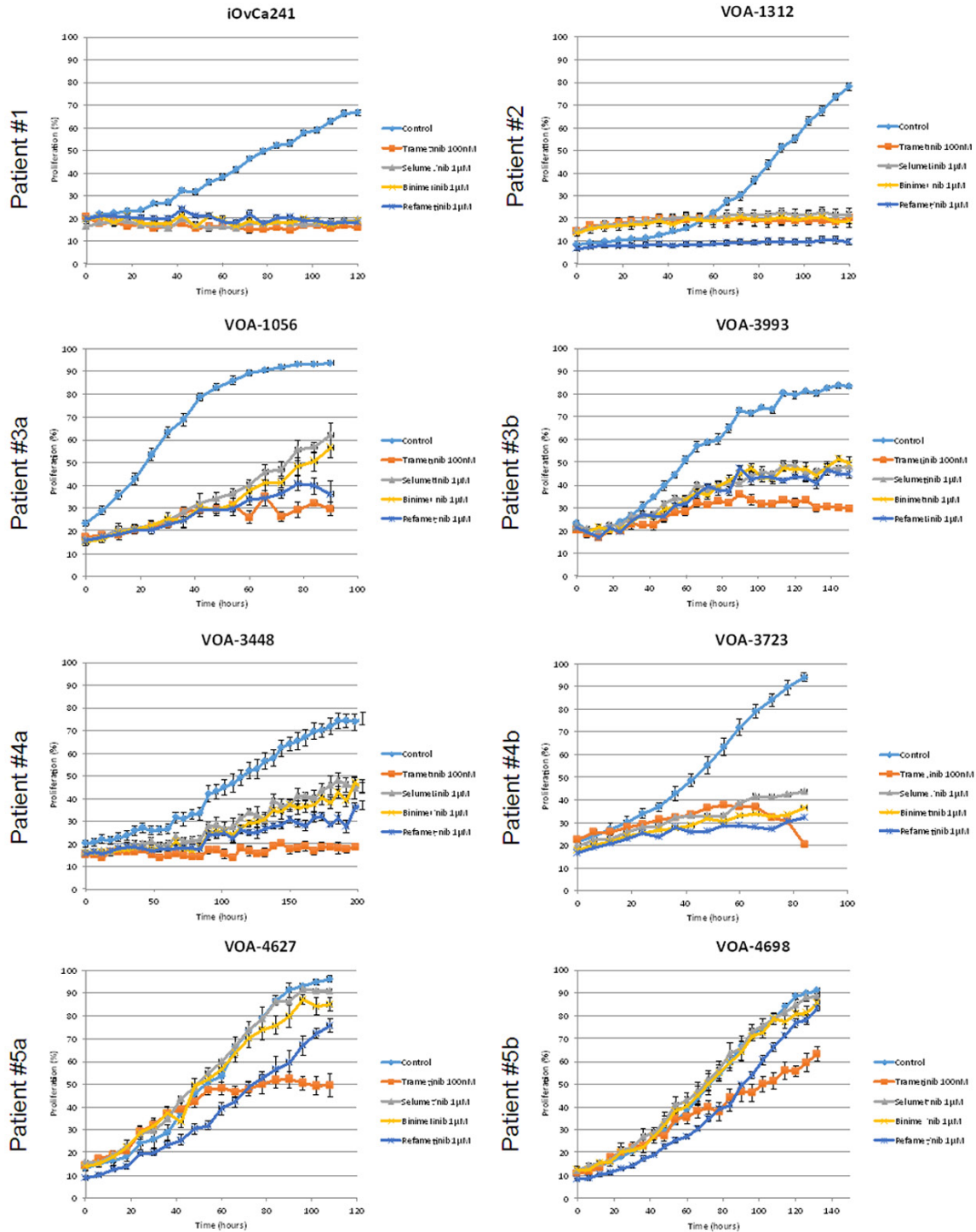


Figure 3. MEKi effects on LGSC cell proliferation. Single dose (at time 0) MEKi proliferation curves for each cell line starting at 20% confluence. Proliferation was measured using Incucyte®. Five replicates were performed for each experimental condition. Experimental results were confirmed by independent experiments performed in duplicate.

the degree of on-target efficacy by measuring the levels of ERK1/2 phosphorylation (p-ERK1/2), a downstream effector of MEK. To

analyze p-ERK1/2 we used two protein-based techniques: Western blot (WB) and capillary isoelectric point focusing technology (cIEF). As

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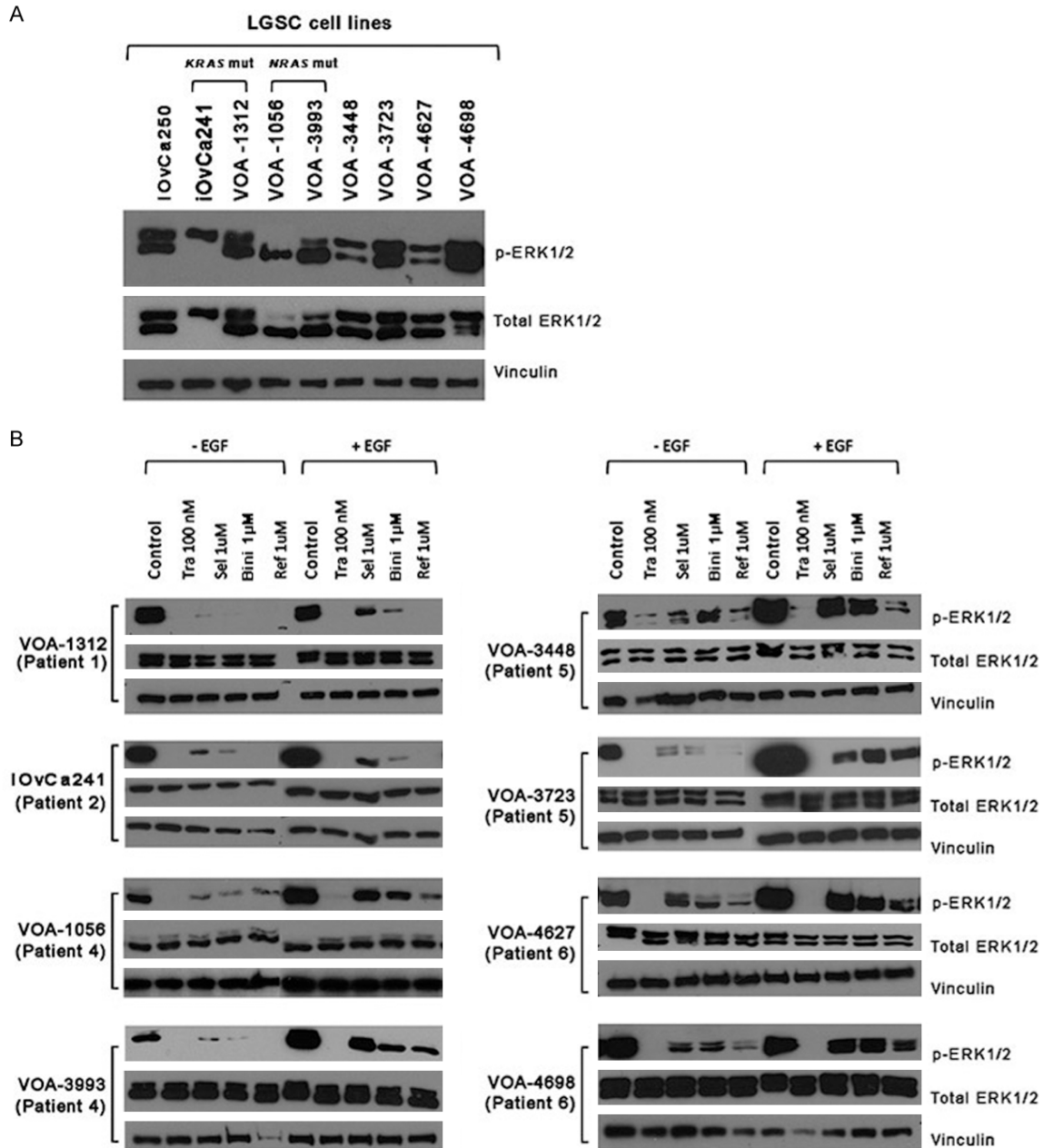


Figure 4. MEKi on-target effects on ERK1/2 phosphorylation in LGSC cells. A: Baseline ERK and p-ERK1/2 by western blot is shown for each cell line. B: ERK and p-ERK1/2 levels in response to MEKi treatment. Tra = trametinib, Ref = refametinib, Sel = selumetinib, Bin = binimetinib. Films were intentionally overexposed to detect signal in lanes with low p-ERK1/2 expression. For each cell line all treatment conditions were performed as part of the same experiment. All experimental findings were confirmed in independent experiments performed in triplicate. Note: membranes incubated with p-ERK1/2 antibody were overexposed to capture all remaining signal after MEKi treatment. WB images have been cut to keep the same sample order. All membranes incubated with the same antibody have been exposed for the same period of time.

seen in **Figure 4A**, basal levels of p-ERK1/2 varied among cell lines, being lower in iOvCa241, VOA-1056, VOA-3448, VOA-4627, and higher in iOvCa250, VOA-1312, VOA-3723, VOA-3993, and VOA-4698. Of note, iOvCa241

cells only expressed the ERK1 isoform (ERK2 homozygous copy loss was detected in the CNV array), and VOA-1056 cells predominantly expressed ERK2. Interestingly, an increase in p-ERK1/2 was detected in all three of the recur-

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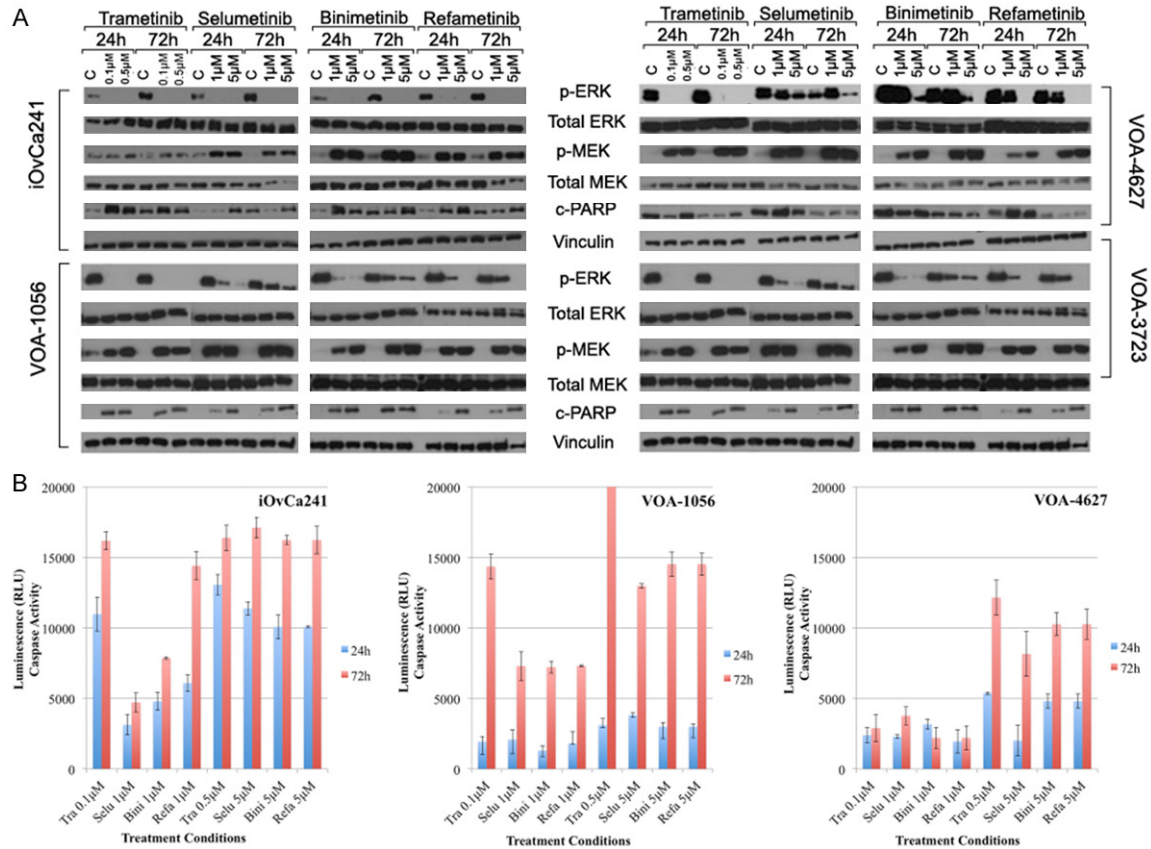


Figure 5. Time course and dose-response effects of MEKi treatment in LGSC cell lines. A: WB expression levels of ERK1/2, MEK and PARP after MEKi treatments with different dosing (low versus high) over time (24 h versus 72 h). B: Apoptosis induction measured by caspase 3/7 after MEKi treatment in LGSC cell lines. All experiments were repeated. Note: Caspase 3/7 control measurements at 24 h and 72 h were subtracted from all treatment values in the graphs shown.

rent cell line samples compared to their patient-matched initial cell line samples (VOA-1056 vs VOA-993; VOA-3448 vs. VOA-3723; VOA-4627 vs. VOA-4698). This increase in p-ERK1/2 was observed over time independently of patient treatment.

We then treated each cell line with the different MEKi (trametinib 0.1 µM, selumetinib 1 µM, binimetinib 1 µM, or refametinib 1 µM), and analyzed the levels of p-ERK1/2 expression at 24 hours. For most cell lines, 1 µM selumetinib or binimetinib only partially inhibited p-ERK1/2, there was greater inhibition using 1 µM refametinib, and complete inhibition with 0.1 µM trametinib (**Figure 4B**). EGF treatment was used to determine if each MEKi could still suppress phosphorylation after MAPK pathway stimulation. Only trametinib was able to completely block the induction of p-ERK1/2. To further

characterize drug effect on p-ERK1/2 isoforms on native protein lysates, we selected 4 cell lines for further study using cIEF: the sensitive iOvCa241 cell line, and one cell line from each of the paired samples (VOA-1056, VOA 3723, and VOA-4627). The results from these experiments are shown in [Supplementary Figure 3](#). LGSC cells treated with 0.1 µM trametinib showed little detectable levels of p-ERK1/2 across cell lines. Refametinib (1 µM) was the second most effective inhibitor of p-ERK1/2. Using cIEF we could not observe any drug-related differences in p-ERK1/2 in the treated sensitive cell line (iOvCa241) as treatment with any of the MEKi resulted in complete inhibition of p-ERK1/2 signal. Only partial inhibition of p-ERK1/2 was observed after treatment of the resistant cell lines using 1 µM selumetinib or binimetinib. As observed by WB, iOvCa241 cells only expressed ERK1, and VOA-1056 cells predominantly expressed ERK2.

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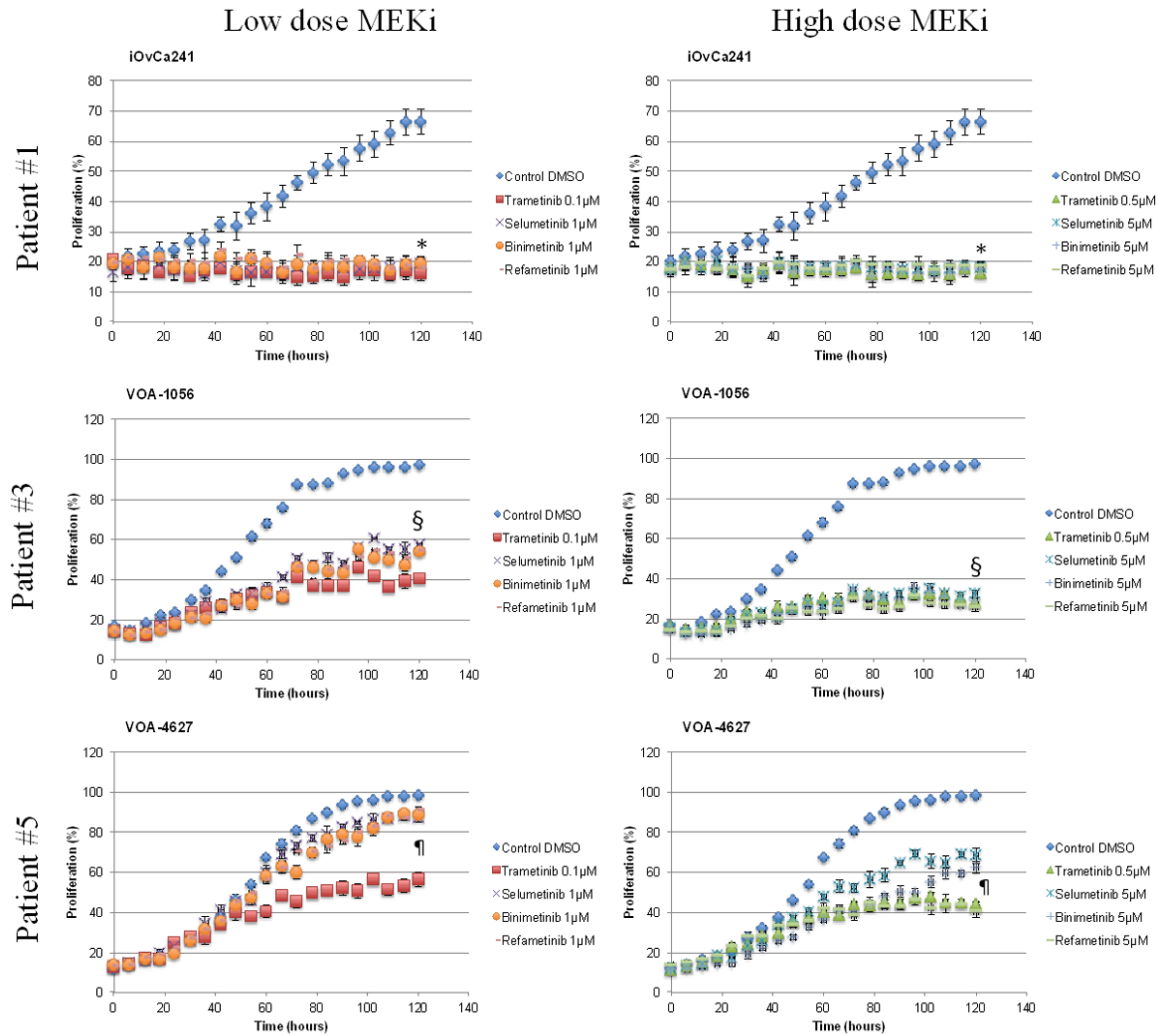


Figure 6. Anti-proliferative effects of MEKi treatment (low and high dose) in LGSC cells. Proliferation experiments showing the effects of low dose (1 \times) and high dose (5 \times) of MEKi treatment (trametinib, selumetinib, binimetinib, refametinib) in three different LGSC cell lines. (*) P -value >0.5 comparing high dose versus low dose for each drug in the iOvCa241 cell line. (§) P -value <0.05 comparing high dose versus low dose for each drug, except trametinib (p -value = 0.09), in the VOA-1056 cell line. (¶) P -value <0.05 comparing high dose versus low dose for each drug in the VOA-4627 cell line.

MEK inhibitor anti-proliferative effects correlate with MEKi on-target efficacy over time

To further explore the inhibitory effects of MEKi treatment in LGSC cells, we performed dose-response experiments on the 4 selected cell lines. Each cell line was treated with low (0.1 or 1 μ M) and high (0.5 or 5 μ M) doses of each MEKi for periods of 24 and 72 hours. p-ERK1/2 expression levels were measured as a marker for MEK activity. In the sensitive iOvCa241, all drugs completely inhibited p-ERK1/2 at any dose or time (Figure 5A). In the other 3 cell lines (VOA-1056, VOA-3723, and VOA-4627) there were similarities in drug-dependent sup-

pression of p-ERK1/2 levels. Trametinib completely blocked p-ERK1/2 even at low dose (0.1 μ M) over 72 hours, however selumetinib or binimetinib at high dose (5 μ M) failed to completely inhibit p-ERK1/2. In contrast, refametinib showed complete inhibition of p-ERK1/2 when used at 5 μ M over 72 hours. In general, MEKi treatment resulted in an increase in MEK1/2 phosphorylation (p-MEK1/2). Interestingly, in the sensitive iOvCa241 line, no clear increase was observed after trametinib treatment.

We then examined the effects of high-dose MEKi treatment on cell proliferation and

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p-ERK1/2 expression. In iOvCa241, higher dosing (**Figure 6**) did not further reduce proliferation rates, as low doses completely inhibited proliferation ($P > 0.5$; t-test, high vs. low dose of each drug; 120 hours post-treatment). Notably, in the 3 resistant cell lines (VOA-1056, VOA-3723, and VOA-4627) there were drug-dependent differences in proliferation after higher drug dosing. For example, in the most resistant cell line, VOA-4627, each MEKi significantly decreased proliferation rates 120 hours post-treatment ($P < 0.05$; t-test, high dose vs. low dose of each drug) (**Supplementary Table 4**). In contrast, for VOA-1056, statistically significant decreases in proliferation were observed at 120 hours post-treatment for all drugs ($P < 0.05$; t-test) except trametinib ($P = 0.09$; t-test, high dose vs. low dose). In 3 more resistant cell lines, dose dependent reductions in p-ERK1/2 over time (**Figure 5A**) correlated with degree of anti-proliferative effect for all drugs except trametinib (**Figure 6**). Once again, trametinib and refametinib more effectively blocked p-ERK1/2 and show the greatest anti-proliferative activity across all tested LGSC cell lines.

MEKi treatment induces apoptosis and the degree of apoptosis correlates with drug sensitivity

Using the same low and high doses of each MEKi, we then analyzed the levels of PARP activation (cleaved PARP or c-PARP) in four LGSC cell lines by WB (**Figure 5A**). Three cell lines (iOvCa241, VOA-1056 and VOA-3723) had little or no detectable baseline c-PARP activity, but considerable c-PARP induction after MEKi treatment. In contrast, VOA-4627 showed considerable baseline PARP activity without a corresponding increase after MEKi treatment. As another marker for apoptosis activation, we also measured the level of caspase 3/7 cleavage in 3 cell lines (iOvCa241, VOA-1056, and VOA-4627). **Figure 5B** shows the changes in cleaved caspase 3/7 (above subtracted baseline control) pre-treatment and 24 and 72 hours post-treatment. All MEKi increased cleaved caspase levels at 24 and 72 hours. The MEKi sensitive iOvCa241 cell line showed the most robust apoptotic response to MEKi treatment and trametinib at low dose was as effective at inducing apoptosis as the high dose of any other MEKi (**Figure 5B**). The MEKi resistant VOA-4627 cell line showed no difference in caspase activity when treated with lower doses

of MEKi over time ($P = 0.08$; t-test: 24 vs. 72 hours for all MEKi). In contrast, the less resistant VOA-1056 cells, showed significant increases in cleaved caspase after 72 h treatment ($P < 0.00001$; t-test 24 vs. 72 hours, for all MEKi).

Discussion

We have established and molecularly characterized a cohort of advanced/recurrent LGSC cell lines. LGSC cells are difficult to culture and only a few LGSC cell lines (MPSC-1, HOC-7) are available and studied to date [24, 34, 35]. Clinical and molecular characterization (pathology at collection, tumor stage, treatment status, STR profiling, mutation and CNV analyses) of cell lines provides a basis for using these lines as experimental models. For example, previous tumor mutational studies have shown that *BRAF* mutations are found in LGSC, but are mostly confined to early-stage cases [30, 36, 37]. As such cases are often cured by surgery, the use of *BRAF* mutant LGSC cell lines for investigational drug studies will be of limited value. No *BRAF* mutations were identified in our cell line cohort in agreement with a previous study [38], though there has been one reported patient with a *BRAF* fusion who achieved a complete response to binimetinib in combination with paclitaxel [39]. *KRAS/NRAS* mutations are relatively frequent in advanced/recurrent LGSC [16], and in our study they were detected in 5 out of 10 cell lines from 4 different patients. Further study of LGSC cell lines with *KRAS/NRAS* mutations is very important to understand how these mutations affect pathway signalling and responses to MAPK pathway inhibition in LGSC. Successful targeted therapies against *KRAS* mutations have proven to be challenging [40, 41] and it is not clear whether MEKi will effectively treat LGSC patients with *KRAS* mutations [23].

Cancer hotspot mutational analysis revealed that cell lines derived from tumor samples obtained from the same patient at different times during the course of the disease shared the same mutations (see mutations in cell line pairs VOA-1056 and VOA-3993; VOA-3348 and VOA-3723; and VOA-4627 and VOA-4698; **Table 2**). Copy number analysis showed marked heterogeneity among LGSC cell lines. Further genomic disruption was seen over time, or in

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response to treatment, in the paired cell lines that we analyzed, VOA-1056 and VOA-3993. Despite this heterogeneity, LGSC cell lines shared common regions of copy number change. Loss of chromosome 9p was detected in all cell lines, an event that has been observed in other recent genomic profiling studies done in LGSC tumors [42-44]. This genomic region is known to contain the *CDKN2A/B* locus, which encodes for three well-known tumor suppressor genes: p14 (Arf), p15 (INK4b), and p16 (INK4a). Loss of p16 has been described in LGSC tumors in comparison to their high-grade counterparts [2, 45]. It is of interest that loss of chromosome 9 is frequently seen in LGSC but is rare in SBOT, suggesting a potential role for malignant transformation [42, 43]. Other copy number changes were observed among cell lines included gains in chromosomes 8, 12 and 20. Gains in chromosome 12 have been described in both LGSC and SBOT [43, 46]. Notably, *KRAS* is located on chromosome 8. More in-depth study of these regions may help further characterize LGSC in patients with relapsed or progressive disease.

We used our LGSC cell lines to test four MEKi and noted remarkable differences in the sensitivity of these lines to the individual drugs tested. We noted that the proliferation assays were helpful in terms of classifying sensitive versus resistant cells as the results from the IC50 experiments did not well characterized the efficacy of the different drugs over time. Based on patterns of growth inhibition and apoptosis, we identified two sensitive cell lines (VOA-1312 and iOvCa241). These two lines demonstrated marked inhibition of proliferation in response to all MEKi tested even at low doses. The iOvCa241, MEKi sensitive cells, also showed a marked apoptotic response after MEKi treatment when compared to two MEKi resistant cell lines (VOA-1056 and VOA-4627) (**Figure 5**). The remaining cell lines show varying degrees of resistance to MEKi with less anti-proliferative effects in response to drug. In most MEKi resistant cell lines, though a dose-response effect on proliferation was observed, proliferation continued despite higher doses of drug (See **Figure 3**). To confirm the significance of these findings it will be important to test additional LGSC cell lines derived from patients known to be sensitive or resistant to MEKi treatment.

Our evaluation of the biological effects of four different MEKi in LGSC cells showed that following a single treatment, trametinib is a more effective inhibitor of cell proliferation at 10-fold lower doses than the other drugs tested (refametinib, selumetinib, and binimetinib). Trametinib also showed the greatest reduction in cell proliferation and viability in the more resistant cell lines. These observations are similar to recent reports of MEKi testing in lung cancer cell lines and tumor models [32, 47]. Trametinib most effectively induced apoptosis in our cell lines, which has been described as an important mechanism of MEKi action [31].

The study of on-target effects of each MEKi in our LGSC cell lines revealed interesting differences relating to cellular p-ERK1/2 at baseline and in response to drug treatment. In our study, there was no correlation between baseline p-ERK levels and MEKi sensitivity in keeping with a previous report [33]. We did observe a remarkable correlation between the degree of inhibition of p-ERK1/2 levels and the anti-proliferative effects of each MEKi. Interestingly, we observed that binimetinib, as well as selumetinib, was much less effective at inhibiting p-ERK1/2 and cell proliferation in LGSC cell lines than trametinib or refametinib. Dose dependent inhibition of p-ERK1/2 was observed in our study for all drugs except trametinib, which effectively blocked p-ERK1/2 even at low doses (0.1 μ M). This finding is in keeping with a study on breast cancer cell lines showing no correlation between trametinib IC50 values and inhibition of ERK signaling as p-ERK1/2 is inhibited at trametinib concentrations not affecting proliferation [33]. Our results therefore suggest that using a particular MEKi dosing schedule or drug that fails to eliminate p-ERK1/2 will not result in maximal anti-proliferative effects in tumors from LGSC patients. On the other hand, cells with intrinsic resistance to MEKi continue to proliferate despite marked inhibition of p-ERK1/2. This observation was most apparent using trametinib as it so effectively inhibits ERK phosphorylation at low concentrations. Recently, an investigational drug trial using binimetinib in LGSC was closed following an interim analysis that failed to show a pre-defined benefit on progression-free survival compared to chemotherapy. A large randomized trial is currently open (NCT02101788) to evaluate trametinib as a

treatment for LGSC and this will be a very informative trial relating to the use of MEKi in this disease.

In 2012, an important phase 2 clinical trial was reported evaluating selumetinib as a treatment for recurrent LGSC. There was a 15% response rate and *KRAS/BRAF* mutations were not a predictor of response. Copy number changes or other MAPK-pathway related aberrations were not evaluated. As clinical responses to selumetinib were infrequent, it will be important to identify biomarkers of MEKi response in order to treat those patients who are most likely to benefit. In the present study, the most sensitive cell lines (VOA-1312 and iOvCa241) were found to have mutations in *KRAS* (G12V and G12D respectively) with associated gene copy-number gains. This finding suggests that these lines may be highly dependent on *KRAS* activity. Polh *et al.* found that primary ovarian cancer cell lines and cultures with *BRAF* (4 cell lines) and *KRAS* mutations (2 cell lines) were more sensitive to MEKi (CI-1040) than 6 wild type lines [24]. In a study of colon cancer cell lines, MEKi treatment impaired anchorage-independent growth of nearly all *KRAS/BRAF* mutants, but not wild type cells. However, neither increased baseline p-ERK1/2 nor a reduction of p-ERK1/2 secondary to MEKi treatment correlated with inhibition of anchorage independent growth [48]. Recently, it has been suggested that *KRAS* mutations may in fact result in a hyper-excitable *KRAS* rather than an altered protein in a statically active state [41]. MAPK signaling is very complex as there are copy number changes, other pathway mutations, and cross talk mechanisms all of which can affect MAPK signaling and the response to MEKi drug treatment.

This study shows marked differences in LGSC cell line MEKi efficacy. We observed that trametinib is highly effective in blocking p-ERK1/2 at concentrations ten-fold lower than the other MEKi, and this on-target effect is reflected in its *in vitro* drug efficacy. As effective systemic treatments are lacking for this disease and no xenograft models for pre-clinical testing are available, these cell lines should be used to better understand drug- or tumor-related determinants of MEKi sensitivity. There is a need for this work as investigational MEKi (trametinib and pimasertib) are currently being tested in large randomized trials designed for drug regis-

tration and approval. These trials are proceeding with a very limited understanding of MAPK pathway function in LGSC. With recent advances in molecular medicine, pre-clinical investigational drug testing can play an important role in the design of clinical trials, molecular companion studies, and the selection of the most promising investigational drugs for clinical testing. Linking molecular research strategies with drug development and clinical trial design will be necessary in order to improve treatment outcomes in rare cancer types such as LGSC.

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Disclosure of conflict of interest

None.

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