Pharmacodynamic Effects of Seliciclib, an Orally Administered Cell Cycle Modulator, in Undifferentiated Nasopharyngeal Cancer

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Abstract

Purpose: Cell cycle dysregulation resulting in expression of antiapoptotic genes and uncontrolled proliferation is a feature of undifferentiated nasopharyngeal carcinoma. The pharmacodynamic effects of seliciclib, a cyclin-dependent kinase (CDK) inhibitor, were studied in patients with nasopharyngeal carcinoma.

Experimental Design: Patients with treatment-naïve locally advanced nasopharyngeal carcinoma received seliciclib at 800 mg or 400 mg twice daily on days 1 to 3 and 8 to 12. Paired tumor samples obtained at baseline and on day 13 were assessed by light microscopy, immunohistochemistry, and transcriptional profiling using real-time PCR low-density array consisting of a panel of 380 genes related to cell cycle inhibition, apoptosis, signal transduction, and cell proliferation.

Results: At 800 mg bd, one patient experienced grade 3 liver toxicity and another had grade 2 vomiting; no significant toxicities were experienced in 13 patients treated at 400 mg bd. Seven of fourteen evaluable patients had clinical evidence of tumor reduction. Some of these responses were associated with increased tumor apoptosis, necrosis, and decreases in plasma EBV DNA posttreatment. Reduced protein expression of Mcl-1, cyclin D1, phosphorylated retinoblastoma protein pRB (T821), and significant transcriptional down-regulation of genes related to cellular proliferation and survival were shown in some patients posttreatment, indicative of cell cycle modulation by seliciclib, more specifically inhibition of cdk2/cyclin E, cdk7/cyclin H, and cdk9/cyclin T.

Conclusions: Brief treatment with this regimen of seliciclib in patients with nasopharyngeal carcinoma is tolerable at 400 mg bd and associated with tumor pharmacodynamic changes consistent with cdk inhibition, and warrants further efficacy studies in this tumor.

A common feature of cancer is dysregulated cell cycle control that allows uncontrolled cell cycle progression through events that promote global gene transcription (1). These defects could be a result of activation of cyclin-dependent kinases (cdk) 4 and 6 or their regulatory D-type cyclins, loss of function of cdk inhibitors like $p16^{INK4A}$, $p27^{kip1}$, loss of activity of p53, or excessive activity of cdk4/6. Whereas cdk4 and 6 and cdk2 when associated with their cyclins are known to activate S-phase DNA replication through retinoblastoma protein phosphorylation and consequent unbridled transcriptional effects of E2F, CDK7/9 are able to maintain gene transcription by activating RNA polymerase II (1–3). It is known that cancer cells require continuous expression of antiapoptotic genes for maintenance of their transformed state (4). Therefore, down-regulation of gene transcription through inhibition of CDK2, CDK7/9 would result in apoptosis. Cell cycle inhibitors exploit this mechanism as potential anticancer drugs.

Seliciclib (Cyclacel Ltd.; CYC202; R-roscovitine), an orally administered small molecule inhibitor of the serine/threonine kinases CDKs, through competition with ATP for binding, can induce apoptosis in several human tumor cell lines, including nasopharyngeal carcinoma, through inhibition of retinoblastoma and RNA polymerase II carboxy terminal domain phosphorylation, leading to a reduction in levels of key antiapoptotic proteins like Mcl-1 and XIAP, and cell cycle regulators such as cyclin D1 (5–7). The IC_{50} of seliciclib for the various cdks are in similar ranges [cdk2/cyclin E (0.1 μmol/L), cdk7/cyclin H (0.36 μmol/L), and cdk9/cyclin T (0.8 μmol/L); ref. 8]. Seliciclib is currently in phase II clinical trials in combination with chemotherapy in breast and non–small cell

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Translational Relevance

Cell cycle inhibitors have not been shown previously to induce molecular changes *in vivo* in solid cancers. Using a window design clinical study, we showed that seliciclib down-regulated transcription of genes relevant to EBV-mediated tumorigenesis and control of apoptosis through activation of signaling pathways that included mitogen-activated protein kinase, c-Jun NH_2-terminal kinase, p38, Janus kinase/signal transducer and activator of transcription, nuclear factor κB, phosphoinositide 3-kinase/protein kinase B, and RhoGTPases in nasopharyngeal cancer, where EBV-mediated transformation and expression of latent membrane protein 1 has been associated with carcinogenicity. In addition, nasopharyngeal carcinoma is characterized by dysregulation of the cell cycle, particularly through overexpression of cyclin D1 and silencing of $p16^{INK4A}$, promoting gene transcription. Therefore, inhibition of these pathways potentially restores cell cycle regulatory events. This proof of principle provides the evidence required for a formal evaluation of cell cycle inhibitors in nasopharyngeal cancer, and represents a significant advance in the application of novel molecular-targeted agents to therapy of this cancer.

lung cancer, but its pharmacodynamic effects of cdk inhibition by itself in solid tumors *in vivo* have not been shown. In chronic lymphocytic leukemia cells *in vitro*, seliciclib has been shown to induce apoptosis through the down-regulation of genes, and a similar mechanism of apoptosis has been described with another cdk inhibitor flavopiridol (9).

Undifferentiated nasopharyngeal cancer is an aggressive disease common in SouthEast Asia, and although concurrent chemoradiotherapy has improved the outcome of locally advanced disease, toxicities associated with definitive treatment and the poor outcome of recurrent disease with chemotherapy necessitate the development of more effective and less toxic alternatives. Cell cycle dysregulation is frequently found in nasopharyngeal carcinoma, in which activating cyclins such as cyclin D1 are often overexpressed whereas cyclin-dependent kinase inhibitors such as $p16^{INK4A}$ are down-regulated (10–12). This promotes association between cyclin D1 with cdks 4 and 6 to facilitate G_1 progression through phosphorylation of retinoblastoma, thereby promoting E2F-mediated gene transcription (1). These cell cycle defects have significance for treatment, as restoration of p16 expression or inhibition of cyclin D1 by small interfering RNA reduces proliferation of nasopharyngeal carcinoma cell lines (13).

On this basis, we conducted a clinical study of seliciclib in patients with untreated nasopharyngeal carcinoma to determine its tolerability, pharmacokinetics, and clinical as well as cellular effects.

Patients and Methods

Patients with treatment-naïve, localized, histologically confirmed WHO type III undifferentiated nasopharyngeal carcinoma were enrolled. Other eligibility criteria included age ≥21 y, Eastern Cooperative Oncology Group performance status of 0 or 1, and adequate bone marrow, renal, and hepatic function. Patients were excluded if they were on alternative medications, had uncontrolled major medical conditions, were pregnant or lactating, had evidence of malabsorption, had other malignancies within 5 y, or were known carriers of hepatitis B or C or HIV. The protocol was approved by the local institutional review board and all patients gave written informed consent prior to participation.

Treatment, procedures, and methodology. Baseline history, physical examination, performance status, biochemical profile, and complete blood count were done within 7 d before starting treatment. Patients had endoscopic assessment of tumor with tumor biopsy within 72 h prior to commencing seliciclib and on day 13 of treatment. Seliciclib was supplied by Cyclacel Ltd in 200-mg capsules. Previous phase I studies have established that 800 mg/m^2 twice daily for 7 d is associated with dose-limiting toxicities; 5 d continuous dosing seemed to be tolerable (14). Therefore, patients were orally administered a starting dose of 800 mg bd at least 2 h postprandial on days 1 to 3 and 8 to 12 for a single 14-day cycle. Treatment of three patients was planned at this dose level 1 with an additional three patients to be treated if one patient had dose-limiting toxicities. Further enrollment at level 1 would continue if no more than two of six patients had dose-limiting toxicities. Reduction to dose level 2 was mandated if more than one of the first three patients exhibited dose-limiting toxicities. Patients were seen on days 8 and 12 for repeat laboratory testing, assessment of treatment compliance, and toxicity according to National Cancer Institute Common Toxicity Criteria version 2.

Tumor measurements and assessments. Bidimensional tumor measurements were done on all palpable cervical lymph nodes at pretreatment and on days 8 and 12 using skin calipers. The sum of the products of the pretreatment measurements was subtracted from the sum of the product of the posttreatment measurements and divided by the sum of the product of the pretreatment measurements. Patients with >25% decrease in the sum of the product of the posttreatment measurements were considered to show clinical response. Computed tomography scans of the nasopharynx and neck were done within 2 wk of starting seliciclib and immediately following treatment with seliciclib for radiotherapy planning, and tumor measurements were made according to the Response Evaluation Criteria In Solid Tumors.

Immunohistochemistry and histologic examination. Tumor tissue was assessed for the following biomarkers: cyclin D1, Mcl-1, phosphorylated retinoblastoma-Ser807/811 (cdk4-specific phosphorylation site) and pT821 (cdk2-specific phosphorylation site), Ki67 expression, and apoptosis (terminal deoxynucleotidyl transferase biotin-dUTP nick end Labeling or TUNEL staining). Light microscopy was used to evaluate histologic changes using H&E staining.

After deparaffinization and rehydration, antigen retrieval was done by boiling in citrate buffer in a pressure cooker (Milestone, T/T Mega). Endogenous peroxidase activity was blocked with incubation in 0.03% hydrogen peroxide/sodium azide buffer for 5 min. Primary antibody was then applied at appropriate dilution overnight at room temperature [anti-phospho-retinoblastoma (Ser807/811) antibody, 1:300, Cell Signaling Technology Inc.; anti-phospho-retinoblastoma (pT821) antibody, 1:500, Biosource International Inc.; cyclin D1, 1:100, Dako; Mcl-1, 1:200, Labvision Corporation; Ki-67, 1:200, Labvision Corporation]. Detection of bound antibody was accomplished with the DAKO Envision TM+ System (DAKO). After incubation with primary antibody, slides were rinsed with 0.0002% Tween 20 solution and incubated for 30 min with horseradish peroxidase–labeled polymer conjugated to antimouse or antirabbit secondary antibody. Following 0.0002% Tween 20 rinses, sections were developed with 3,3′-diaminobenzidine for 3 min, counterstained with Gills hematoxylin (Merck), and mounted with DPX (BDH Chemicals). TUNEL staining was done using Promega's DeadEnd Colorimetric TUNEL system per manufacturer's protocol.

The pretreatment and posttreatment samples were assessed for extent and intensity of staining using a semiquantitative score of 0 to

3, and then compared and reported as increased expression, decreased expression, or maintenance of the expression. Only those cases with a clear, unequivocal difference leading to up- or down-regulation were scored as such, to avoid possible false positives in borderline cases. Scoring was done by two independent pathologists who were blinded to the timing of sample collections. After examination of all the collected paired pretreatment and posttreatment samples, only those in which it was perceived that the biopsy was deep enough to represent tumor primarily (as opposed to adjacent surface epithelium) were considered suitable.

Low-density array real-time reverse transcriptase-PCR. Total RNA from snap-frozen tumor samples was extracted using Tri-Reagent (Molecular Research Centre Inc.). Total RNA yields and purity were determined using the ND-100 Spectrophotometer (Nanodrop Technologies). Public databases and publications were extensively reviewed to identify 380 genes involved in pathways of cell proliferation, cell cycle regulation, and apoptosis. Real-time reverse transcription-PCR analysis was carried out using the Micro-Fluidic Cards system (Applied Biosystems), preloaded with inventoried TaqMan Gene Expression Assays for the selected genes according to the manufacturer's instructions. Briefly, 1.2 μg of total RNA was reverse transcribed into cDNA using the cDNA Reverse Transcription Kit (Applied Biosystems). Ten microliters of cDNA sample were mixed with 490 μL of nuclease-free water and 500 μL of 2 × PCR master mix. One hundred microliters of the reaction mix were loaded into each of the eight sample ports on the Micro-Fluidic Cards. Thereafter, the cards was centrifuged twice for 1 min at 331 × *g* at room temperature and then sealed and analyzed on the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). Relative gene expression levels were determined by SDS 2.2.1 software using the ΔCt method (Applied Biosystems). Expression levels of target genes were normalized to GAPDH.

Plasma EBV viral copies. DNA from plasma samples obtained at baseline and on day 12 of seliciclib treatment was extracted using QIAamp Blood Kit (Qiagen). Plasma EBV DNA copy number, a specific validated marker of clinical tumor burden and treatment response in nasopharyngeal carcinoma (15, 16), was quantitated using real-time PCR primers and the probe targeted the BamHI-W region of the EBV genome as previously described (17).

Pharmacokinetic analysis. Pharmacokinetic samples were drawn from all patients at 0, 1, 2, 4, and 6 h on days 1 and 12 of treatment. Plasma levels of seliciclib and its most abundant carboxylated metabolite, PEF30-128, were assayed using a validated liquid chromatography-tandem mass spectrometry method at ACC, Germany. Non-compartmental pharmacokinetic analysis based on the individual patient's area under the concentration-time curves (AUCs) was done using the log-linear trapezoidal option, from time 0 to the last time point of each sampling period as well as from time 0 to infinity. Pharmacokinetic analyses were carried out using Kinetica version 4.4 (Innaphase Corp.).

Statistical analysis. Changes in gene expression for the panel of genes for each patient in the dataset were subjected to unsupervised hierarchical clustering to cluster patients with common gene expression changes. For each gene G, the "relative expression value" e_G between day0 and day13 as e_G = day13/day0 if day13 >day0, and as e_G = -day0/day13 if day0 >day13. Hence eG = 3 means its day13 value is 3 times its day0 value, and eG = -3 means its day13 value is only 1/3 of its day0 value. Only genes that satisfied all of the following: (*a*) have present calls in >80% of samples, (*b*) SD > 0, (*c*) at least one observation has $|e_G| > 2$, and (*d*) the maximum and minimum values differ by at least 2, were retained. Of the 384 genes, only 283 met these conditions. Two-dimensional hierarchical clustering was done on the 283 genes and 14 samples, with similarity metric set to uncentered correlation. Gene expression levels in the patients were compared prior to and after treatment by using the paired Student's *t* test, using $P < 0.05$ as significant. Statistical analyses were done with SPSS version 13.0 (SPSS Inc.).

Results

Twenty patients were enrolled in this study, of which 16 received seliciclib; 4 patients withdrew consent before receiving

Table 1. Patient characteristics, treatment and clinical tumor response

Patient No.	1	*5*	6	*7*	*8*	*9*	10	*14*	15	*16*	17	18	*19*	*20*
Age (y)	56	45	50	39	46	40	44	47	55	55	69	54	54	45
Sex	M	M	M	M	M	M	M	M	M	M	M	M	M	M
Stage*	IVB	IVB	IVB	IVB	III	III	IVB	III	IVB	III	IVB	III	IIA	III
T	3	2	4	1	2	2	4	3	2	1	2	3	2	2
N	3	3	3	3	2	2	3	2	3	2	3	2	0	2
Dose level (mg)	800	400	400	400	400	400	400	400	400	400	400	400	400	400
Clinical Response (% change)	0	-25	19	NC	-29	-32	-8	-33	-19	-58	-18	-16	-30	-59
CT scan response (% change)	-5	-10	0	-12	-12	-15	0	-15	-20	-8	0	+5	-12	-15
Increased Tumor Necrosis (CT scan)	A	A	A	A	A	A	A	A	P	P	A	A	A	A
Tumor Necrosis (histology)												NT		
Baseline (%)	30	0	0	0	0	0	0	60	0	10	0		0	5
Post-treatment (%)	90	0	0	10	10	10	0	80	10	10	0		0	5
% change in EBV copy number	NA	0	-31	-37	+153	NC	+19	+34	-43	+147	+139	+279	0	-25

NOTE: Data for two patients at 800 mg dose who stopped treatment for toxicity not shown. The % change (for response and EBV DNA copy number) was calculated as follows: % change = (post treatment measurement - baseline measurement)/baseline measurement × 100%.
Patients considered responders by clinical measurement of cervical lymph nodes have numbers in bold italics.
Abbreviations: M, male; A, absent; P, present; NA, not available; NT, no tumor tissue seen in specimen; CT, computed tomography.
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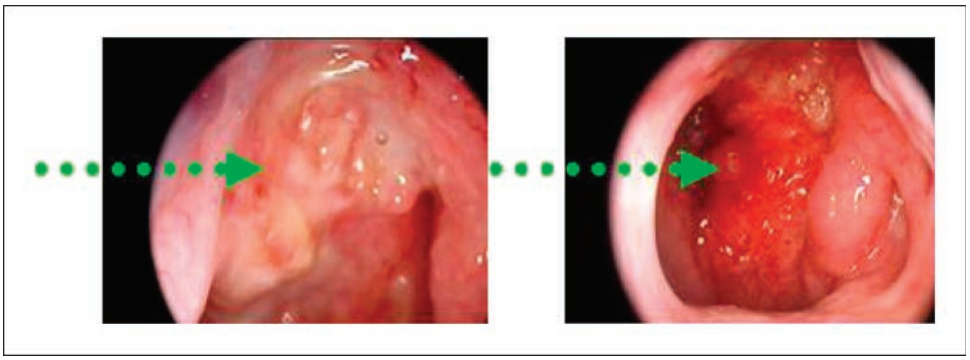

Fig. 1. Fibreoptic nasoendoscopy showing reduction of tumor after 2 wk of treatment with seliciclib. Left, before seliciclib; right, after seliciclib. Arrows point to the tumor in the nasopharynx.

study medication. All 16 patients were male Chinese with a median age of 49 years (range, 40-71 years) with WHO III histological grade, stage II (n = 2), III (n = 6), and IVB (n = 8) disease (Table 1). All patients had localized disease with palpable cervical lymph nodes and were awaiting initiation of definitive treatment with radiotherapy or concurrent chemoradiation.

Toxicity and tumor response. At dose level 1 dose (n = 3), only one patient completed treatment. One patient developed grade 2 nausea/vomiting after the first dose, and the second patient had grade 3 elevation of alanine aminotransferase and cholestatic jaundice on day 8. Hepatitis B and C serologies were negative in this patient. Both patients stopped therapy after the first week. At dose level 2 (n = 13), no side effect, in particular no significant elevation in creatinine or serum transaminases, was observed. Of the 14 evaluable patients, 7 (all on level 2) had >25% reduction in palpable cervical lymph nodes. Corresponding computed tomography scan assessments showed reductions in lymph nodes in the same patients ranging from 5% to 25% (Table 1). All patients had stable disease according to the Response Evaluation Criteria In Solid Tumors for response. Clear reduction in the nasopharyngeal tumor was seen on endoscopy in a patient (Fig. 1). Radiographic evidence of necrosis in the cervical lymph nodes was seen after treatment in an additional two patients at dose level 2.

Serum pharmacokinetics. At the 800-mg bd dose level (n = 3), mean peak plasma concentration of seliciclib reached 6.6 ± 1.5 μg/mL at mean Tmax of 1.3 ± 0.6 hours, resulting in a mean AUC 0-last of 22.8 ± 9.6 μg × h/mL on day 1 (Fig. 2). The mean peak plasma level and AUC 0-last at the 400 mg bd dose (n = 13) was 5-fold lower at 1.3 ± 0.7 μg/mL, and 4.5 ± 2.7 μg × h/mL, respectively. Both AUC 0-∞ (n = 12) and Cmax (n = 13) on days 1 and 12 were similar for both doses.

Pharmacodynamic analysis. One patient at the 800-mg dose level and 13 patients at the 400-mg dose level had paired biopsy samples. The other two patients at the 800-mg dose level did not undergo pharmacodynamic analysis because they stopped dosing in the first week from toxicity.

Immunohistochemistry and histologic examination. Stored tumor samples from the initial patients were found to contain RNA preserving solution, and despite efforts to do antigen retrieval did not yield consistent results; therefore, six paired samples were finally analyzed and results agreed upon by two independent pathologists (Table 2 and Fig. 3). Expression of Mcl-1 and cyclin D1 were clearly decreased posttreatment in three cases, phosphorylated retinoblastoma (pT821) expression was decreased in two samples, whereas Ser 807/811 was unchanged, indicating preferential cdk2 inhibition. Increased apoptosis as measured by TUNEL staining was seen in three patient samples. Ki-67 was decreased in only one patient. The percentage of necrotic area in relation to the overall sample area was calculated; only those cases with a clear, unequivocal difference were considered to have increased necrosis. Six of thirteen suitable paired specimens showed increased necrosis posttreatment (Table 1).

Effect of seliciclib on transcription. Fourteen tumor pairs yielded sufficient RNA and were adequate for analysis of gene expression, all of which contained ≥80% tumor on light microscopy. Unsupervised clustering analysis of all 380 genes showed two clear patient clusters with differential down-regulation of genes (Fig. 4). Greater than 25% of genes showed down-regulation of expression by >25% in the nine patients (5, 7, 8, 9, 14, 16, 17, 18, and 19) in one cluster. Comparison of this cluster of patients with clinical response showed close concordance; therefore this cluster was classified as responders whereas the cluster of five patients whose tumors did not show this pattern of transcriptional repression were classified as nonresponders. To generate hypotheses regarding the cellular

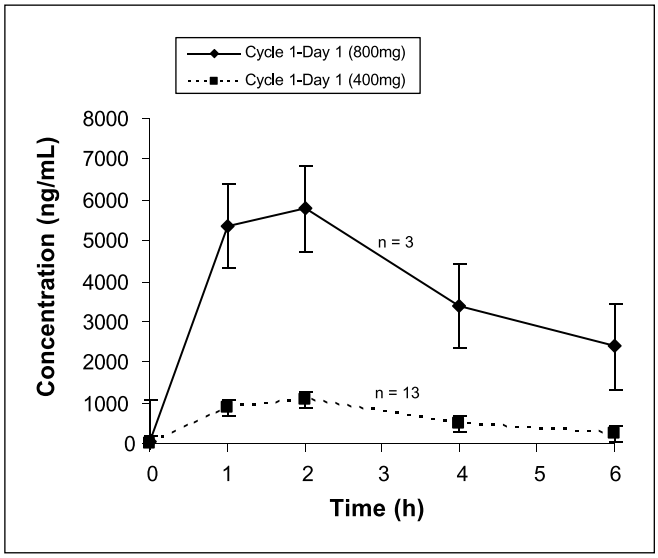

Fig. 2. Plasma disposition curves of seliciclib at 400 mg (n = 13) and 800 mg (n = 3) bd dosing. The mean concentrations at each time point are shown with respective error bars at each time point of sampling.

Table 2. Immunohistochemistry for patients with suitable paired specimens

Patient No.	15	16	17	18	19	20
TUNEL assay	+	+	-/+	na	+	-/+
Mcl-1 staining	-/+	-	-	na	-	-/+
Ki67 staining	+	-	-/+	na	-/+	-/+
Cyclin D-1	-/+	-/+	-	na	-	-
pRB (T821)	-/+	-/+	-/+	na	-/+	-/+
pRB (Ser 807/811)	-	-/+	-	na	-/+	-/+

NOTE: +, increased expression; -, decreased expression; -/+, maintained expression/unchanged expression; na, no tumor tissue seen in specimen.
Abbreviation: pRB, phosphorylated retinoblastoma.

effects of seliciclib exposure, we analyzed the responders for genes that were down-regulated to the greatest extent. This would then yield a panel of most significantly modulated genes at the transcriptional level that could be evaluated against the known cell cycle modulatory effects of seliciclib.

First, the mean gene expression level for all genes of pretreatment and posttreatment samples were compared in the nine responders, and 136 genes were found to be down-regulated posttreatment at $P < 0.05$ using paired t test. Using the GeneCards integrated database (www.genecards.org) and pathway analysis via the KEGG pathway database (www.genome.jp/kegg/pathway.html), 18 of 136 genes were anti-apoptotic, 63 were involved in signal transduction, 41 were involved in the mitogen activated protein kinase (MAPK) pathway, 18 were related to cell proliferation and metabolism, 19 were implicated in RNA synthesis/DNA replication, and 10 were associated with DNA repair. Sixty-three genes represented those with the most significant transcriptional down-regulation ($P < 0.02$), of which the majority could be grouped into pathways involving four major signal transduction pathways: the MAPKs, including extracellular signal-regulated kinase (ERK), p38, and c-Jun NH_2-terminal kinase (JNK); the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway; the nuclear factor κB (NFκB) pathway; and the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathway (Table 3). In addition, genes pertinent to cellular proliferation like heat shock protein 70 (*HSPA8*), involved in folding of signal transduction proteins, protein phosphatase 1 (*pNUTS*), and general transcription factors (*GTF3C4*), DNA repair (*ERCC4* and *ERCC6*, *XPC*), as well as transcription and translation initiation (*eIF2A*) were among the most significantly down-regulated genes (Table 3). Taken together, this shows that seliciclib down-regulates genes that are important for promotion of tumor growth and cell survival in nasopharyngeal carcinoma.

Plasma EBV viral DNA load. Plasma EBV copy number, a marker of disease burden in nasopharyngeal carcinoma, was reduced by =25% in 4 of 13 patients after this brief exposure to seliciclib.

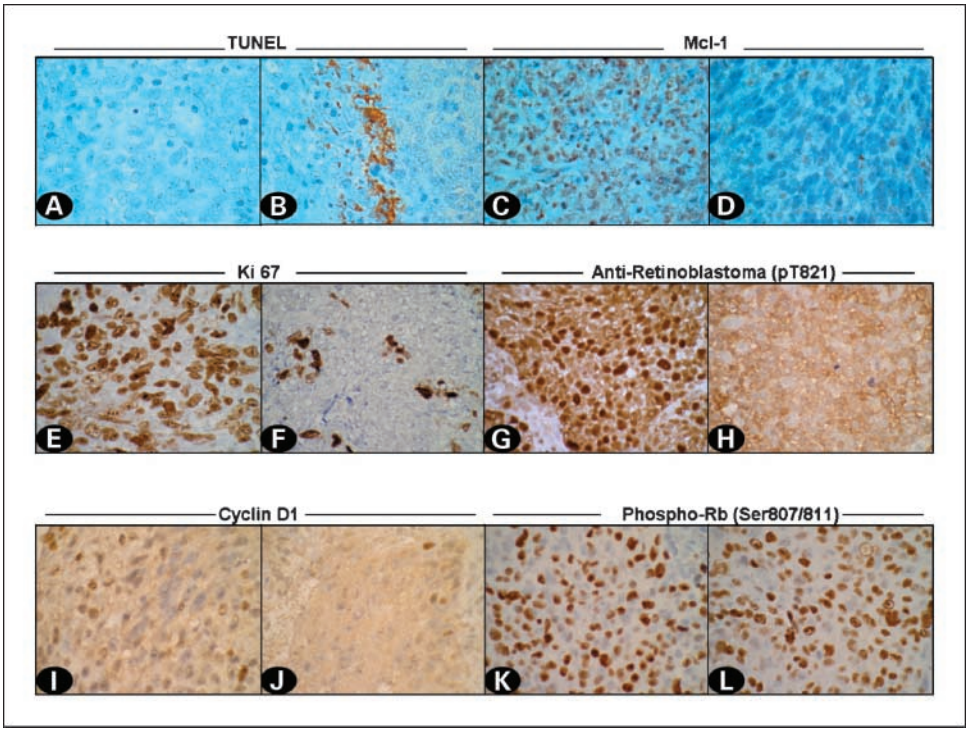

Fig. 3. Representative areas from the samples analyzed. For each given antibody indicated, the first images (*A, C, E, G, I* and *K*) represent pretreatment samples, whereas the second images (*B, D, F, H, J* and *L*) are the corresponding posttreatment. All the images are taken at ×600 magnification.

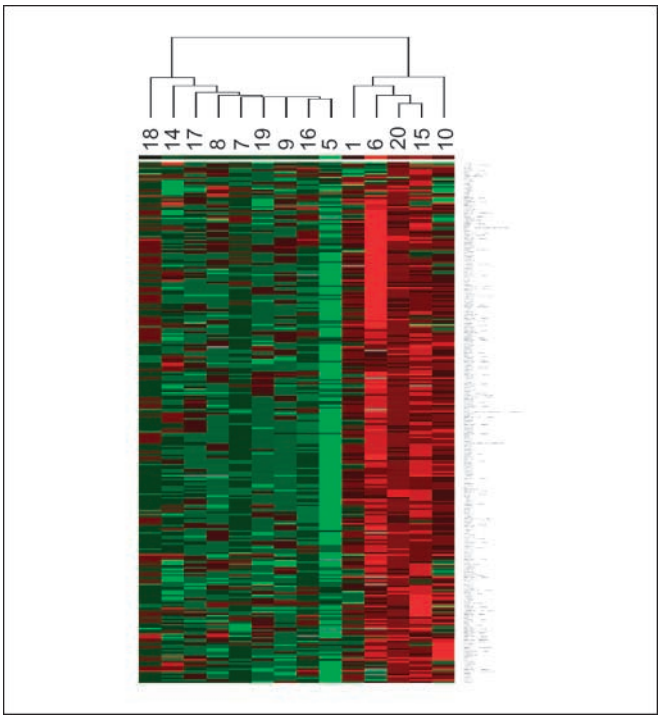

Fig. 4. Effect of seliciclib on gene expression of primary nasopharyngeal carcinoma tumors. Differential expression of the 380-gene panel as measured by real-time quantitative PCR array in patients with nasopharyngeal carcinoma before and after treatment with seliciclib. The cluster was visualized using TREEVIEW. Columns, individual patient samples; rows, individual genes. Color changes within a row indicate changes in posttreatment gene expression levels relative to pretreatment levels: red, up-regulation, green, down-regulation.

Discussion

As development of cytostatic therapeutic agents with specific cellular targets progresses, it is crucial to understand the pharmacodynamic effects of these agents in tumor tissue to guide clinical development. Seliciclib is in phase II clinical trials in combination with chemotherapy; however, its cell cycle inhibitory effects in solid tumor have not previously been shown in patients. Therefore, this study provides the first evidence that seliciclib in clinically tolerable doses exerts effects in tumor cells consistent with cell cycle inhibition, and justifies its clinical development as an anticancer agent in particular in nasopharyngeal carcinoma, in which cell cycle dysregulation such as loss of $p16^{INK4a}$ and overexpression of cyclin D1 could be targeted by cdk inhibition.

In this study, the higher dose of 800 mg bd achieved plasma concentrations that seemed to be higher than previously reported; it was tolerated poorly in the three patients treated, and one patient experienced grade 3 liver toxicity which is a known dose-limiting toxicity of seliciclib, whereas another patient developed grade 2 nausea/vomiting despite optimal antiemetics and diaphoresis. Dose reduction to 400 mg bd was feasible, with no major adverse events. However, the plasma concentrations at this dose resulted in a 5-fold drop in drug exposure. Despite this, antitumor effects were seen as evidenced by lymph node shrinkage after 2 weeks on clinical measurement and computed tomography scans, tumor necrosis/apoptosis on light microscopy, and reduction in plasma EBV DNA in 4 of 13 patients by $\geq$25%, a validated marker of tumor burden in nasopharyngeal carcinoma. Immunohistochemistry of paired samples showed reduced phosphorylated retinoblastoma using a cdk2- but not cdk4-specific antibody, and reduced expression of cyclin D1 as well as Mcl-1, which are known effects of cell cycle inhibition by seliciclib in myeloma cell lines, B-cell chronic lymphocytic leukemia (5, 18). Increase in the plasma EBV DNA after treatment occurred in 6 of 13 patients (from 19% to 279%). The discordance between the EBV viral DNA load and clinical responses could be due to the short 2-week interval between sampling points before and after treatment or assay variability. As plasma EBV viral DNA is mainly derived from tumor, it is possible that tumor apoptosis may result in initial increase in the plasma copy number.

To further elucidate the *in vivo* effects of seliciclib on oncogenic pathways pertinent to nasopharyngeal carcinoma, a panel of genes including those related to apoptosis, cell cycle, cell proliferation, DNA repair, and signal transduction was assessed using quantitative reverse transcription-PCR. Global down-regulation of transcription was observed in most patients studied. The pattern of genes significantly down-regulated were consistent with those previously described in B chronic lymphocytic leukemia cells *in vitro*, including signal transduction, antiapoptotic, cell cycle, DNA repair, and cell proliferation genes. Specifically, antiapoptotic genes like *TRAF5*, *BCL-2*, and *SIRT2* were down-regulated (18). Similarly, down-regulation of DNA repair genes like *XPA*, *ERCC4*, and *ERCC6* may impair cell survival to DNA damaging agents, and may suggest a possible role in combination with DNA damaging agents like cisplatin, an effective agent in treating nasopharyngeal carcinoma.

Of interest, the most significantly down-regulated genes were members of pathways that are known to be activated by latent membrane protein 1 (*LMP1*), an EBV-encoded gene that is known to mediate oncogenic properties by acting as a constitutively active tumor necrosis factor receptor. *LMP1* is known to be expressed in the majority of nasopharyngeal carcinoma cells and is believed to contribute to EBV-mediated tumorigenesis and control of apoptosis through activation of signaling pathways that include the MAPK, JNK, p38, JAK/STAT, NFκB, PI3K/AKT, and RhoGTPases (19, 20). Seliciclib seems to have prominent down-regulatory effect on all these pathways, which may contribute to its positive clinical effects observed in this study. Other down-regulated genes of note were involved in initiation of transcription and translation including *GTF3C4* and *GTF2B/TFIIB*, which may further impair transcription globally, collaborating with other effects to induce tumor cell apoptosis. Phosphatase 1 nuclear targeting subunit (*pNUTS*), a modulator of protein phosphatase 1 that regulates biological pathways like cell proliferation, differentiation, was significantly down-regulated in both cell lines as well as in the patient samples, and may be a critical target in seliciclib-mediated cell cycle inhibition (21).

Taken together, clinical doses of seliciclib can induce conditions that promote apoptosis in nasopharyngeal carcinoma through global transcriptional down-regulation, specifically genes mediating signal transduction via *LMP1* signaling, which is consistent with its ability to inhibit cdk2, 7 and 9, and is in agreement with previously reported findings on chronic lymphocytic leukemia (18).

Interestingly, these molecular perturbations, histologic changes, and clinical responses were seen at the 400-mg bd

dose of seliciclib, which did not achieve plasma concentrations relevant to induce apoptosis in cell lines (IC_{50} 15 μmol/L) although high enough to be relevant for inhibition of CDK2, 7, and 9. It is well established that p-53 is wild-type in tumors from nasopharyngeal carcinoma patients (22–24). *In vitro*, seliciclib is known to be more potent against p-53 wild-type compared with p-53 mutant cell lines (6). In support of this, we have found that in nasopharyngeal carcinoma cell lines, p-53

Table 3. List of genes most significantly down-regulated after seliciclib treatment ($P < 0.02$)

Gene	P	Gene ID	Function
MAPKAPK2	0.00	9261	Signal transduction/MAPK signaling
BID	0.001	637	Proapoptotic
TYMS	0.002	7298	DNA synthesis
RPLP0	0.002	6175	Ribosomal protein
BTRC	0.002	8945	Signal transduction(â-catenin and NF-kB)/transformation
XPC	0.002	7508	DNA repair
HSPA8	0.003	3312	MAPK signaling pathway and cellular macromolecule metabolism
CDK7	0.003	1022	Cell cycle and transcriptional regulation
TBP	0.003	6908	Transcription factor/regulates RNA pol II activity
PIK3C1/PIK3CB	0.00	5291	Signal transduction and transformation
ORC6L	0.003	23594	DNA replication and cell cycle
GADD45A	0.004	1647	Signal transduction/p38-JNK activation
MAPK3/ERK1	0.004	5595	signal transduction/MAPK signaling/apoptosis/cell proliferation
ORC5L	0.00	5001	DNA replication
MCM3	0.005	4172	DNA replication - eukaryotic gene replication initiation
ACBD3	0.01	64746	signal transduction
GTF3C4	0.005	9329	Transcription factor/RNA pol III activity
RAF1	0.005	5894	signal transduction/cell proliferation/interacts with PAKs/MEKs/ERKs
SIRT2/Sir2	0.005	23411	Antiapoptotic (negatively regulates p53 function)
MAP4K1/HPK1	0.006	11184	Signal transduction/MAPK/JNK signaling/apoptosis
SHP2/PTPN11	0.006	5781	Signal transduction/JAK Stat signaling
eIF2A	0.01	83939	Translation initiation
JMJ/JARID2	0.006	3720	Cell proliferation repression
RAB5A	0.007	5868	Signal transduction/cell proliferation
UBE2N	0.007	7334	DNA repair
pNUTS/PPP1R10	0.007	5514	
BCL2L11/BIM	0.007	10018	Proapoptotic
MAP2K5/MEK5	0.007	5607	Signal transduction/MAPK signaling and antiapoptosis
GATA2	0.008	2624	Transcription factor binding
TNFRSF6/FAS	0.008	355	MAPK signaling pathway/apoptosis
ERCC6	0.008	2074	DNA repair
CDC42	0.008	998	MAPK signaling(interacts with PAKs)/cell proliferation
HEAB	0.009	10978	RNA metabolism/ATP GTP binding protein
DVL1	0.01	1855	Signal transduction/b-catenin WNT signaling
SMAD4	0.01	4089	Cell cycle and signal transduction of WNT and TGF? family
DCTD	0.01	1635	Nucleotide metabolism
CYC1	0.01	1537	Proapoptotic
CASP10	0.011	843	Effector of apoptosis
GRB2	0.011	2885	MAPK signaling pathway
MAP2K6/MKK6	0.011	5608	MAPK signaling pathway
HIST4H4	0.012	121504	DNA binding and metabolism
BCL2	0.012	596	Antiapoptosis
STAT1	0.013	6772	Signal transduction
MAPK14/p38	0.013	1432	MAPK signaling pathway
ERCC4	0.013	2072	DNA repair and metabolism
MAX	0.013	4149	MAPK signaling pathway
EP300	0.01	2033	Transcriptional factor/transcription coactivation
MAPK1/ERK	0.013	5594	MAPK signaling pathway/apoptosis
APC	0.014	324	Signal transduction/WNT signaling pathway
MAPK4K4	0.015	9448	MAPK signaling pathway
CRK	0.015	1398	MAPK signaling pathway
FADS1	0.02	3992	Cellular metabolism
PRKACG	0.015	5568	MAPK signaling/aopotosis
ELK4/SAP1	0.017	2005	MAPK signaling and cellular metabolism
PAK2	0.017	5062	MAPK signaling pathway
IKBKB	0.017	3551	MAPK signaling pathway and apoptosis
TP53	0.018	7157	Cell cycle regulation and MAPK signaling pathway
AGTPBP1	0.018	23287	Cellular metabolism
MSK-1/RPS6KA5	0.018	9252	MAPK signaling pathway
BCL11A	0.018	53335	Cellular metabolism
MAP3K6/ASK2	0.018	9064	MAPK signaling pathway/JNK signaling
TRAF5	0.019	7186	MAPK signaling pathway/apoptosis

wild-type HK-1 are more sensitive (IC_{50} 13.8 μmol/L) compared with p-53 mutant CNE-1 cells (IC50 22.1 μmol/L; data not shown). Apoptosis induced by seliciclib via transcriptional down-regulation may be dependent on p53, as previous studies have shown that transcriptional blockade leads to mitochondrial accumulation of p53 and p53-dependent apoptosis (25) via reduction of Hdm2 and stabilization of p53 (26, 27). Furthermore, colon and prostate cell lines with deleted or mutated p53 exhibit decreased sensitivity to apoptosis following RNA polymerase II inhibition (6, 28).

In summary, seliciclib is active in inducing cellular responses in nasopharyngeal carcinoma *in vivo* consistent with cell cycle inhibition at a dose of 400 mg bd, and supports further clinical development of this agent in nasopharyngeal carcinoma, both as a single agent and possibly in combination with cytotoxic agents like cisplatin. Together with the observed cellular effects consistent with known mechanisms of seliciclib, these findings form the basis of an efficacy study of single agent seliciclib in nasopharyngeal carcinoma, which is currently ongoing.

Disclosure of Potential Conflicts of Interest

S. Green, J. Chiao, employment and stock ownership, Cyclacel Pharmaceuticals, Inc.

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