A Phase II Clinical and Pharmacodynamic Study of E7070 in Patients with Metastatic, Recurrent, or Refractory Squamous Cell Carcinoma of the Head and Neck: Modulation of Retinoblastoma Protein Phosphorylation by a Novel Chloroindolyl Sulfonamide Cell Cycle Inhibitor

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ABSTRACT

Purpose: E7070 is a synthetic sulfonamide cell cycle inhibitor that induces hypophosphorylation of the retinoblastoma (Rb) protein and G1 arrest in vitro. This Phase II study was conducted to explore the efficacy, safety, and pharmacodynamics of E7070 in squamous cell carcinoma of the head and neck (SCCHN).

Experimental Design: Patients with metastatic, recurrent, or refractory SCCHN, treated with no more than one prior therapy for recurrent disease, received E7070 at 700 mg/m2 over 1 h every 3 weeks. Pre- and posttreatment tumor fine needle aspirates were subjected to immunohistochemistry with a panel of phospho-specific anti-Rb antibodies. End points included progression-free survival, response rate and duration, overall survival, toxicity profile, and inhibition of Rb phosphorylation.

Results: Because none of the first 15 patients achieved progression-free survival > 4 months, the early stopping rule was invoked. Eleven patients had oropharyngeal cancer and 12 were male. Median age was 59 years (range, 49–73 years). Thirty-nine cycles of E7070 were delivered (median, 2.6 cycles/patient; range, 1–5 cycles). Six patients had stable disease after 2 cycles and 2 patients each subsequently received 1, 2, and 3 additional cycles, respectively, before experiencing progression. Immunohistochemistry of tumor cell aspirates from 3 patients demonstrated reduced Rb phosphorylation posttreatment.

Conclusions: At this dose and schedule, E7070 is unlikely to be superior over single-agent chemotherapy in SCCHN. However, the data suggest that cdk activity can be inhibited in tumor cells, resulting in posttreatment modulation of Rb phosphorylation. In the absence of cytotoxicity, more frequent administration of E7070 may be required to sustain Rb hypophosphorylation and cytostatic growth arrest.

INTRODUCTION

Squamous cell carcinoma of the head and neck (SCCHN) accounts for 4% of all cancers in the United States, resulting in 42,000 cases and 12,000 deaths each year (1). In newly diagnosed, locally advanced disease, the application of aggressive chemoradiotherapy regimens has increased survival at the cost of considerable toxicity (2, 3). Unfortunately, >50% of patients will eventually develop incurable local or metastatic disease. In this population, the role of chemotherapy is palliative, resulting in response rates of 10–15% and median survivals in the range of 4–6 months (4). New targeted approaches are required to improve the outcome of patients with recurrent disease and reduce morbidity in those treated for cure.

Cyclin-dependent kinases (cdks) are core components of the cell cycle machinery that comprise a rational set of targets for novel drug development (5, 6). Orderly transition between the cell cycle phases is governed by cdks, enzymes regulated by phosphorylation, and activated by their association with cyclins. Cyclin D-dependent kinases 4 and 6, as well as cyclin E-cdk2 complexes, sequentially phosphorylate the retinoblastoma protein, Rb, to facilitate the G1-S transition (7). Cyclin A-cdk2 and cyclin B-cdk1 complexes are required for proper S-phase progression and the G2-M transition, respectively. Additionally, two groups of inhibitors, the Cip/Kip and INK4 proteins, also regulate cdk activity (8).

Deregulated cdk activity occurs universally in human cancer (9). Genetic and epigenetic events result in overexpression of cyclins or absent or diminished levels of cdk inhibitors, which
promotes Rb phosphorylation, drives cell cycle progression, and provides tumor cells with a selective growth advantage. In SCCHN, overexpression of cyclin D1 resulting from gene amplification occurs in ~40% of cases, leading to increased activity of cdk4 and cdk6 and loss of growth control. Cyclin E overexpression occurs and is associated with unfavorable clinicopathological parameters. Aberrant expression of cyclin A as well as cdk2 itself has also been described. Reduced p16INK4A expression occurs as a result of gene deletion or inactivation by mutation or methylation. Similarly, low levels of p21Waf1/Cip1 and p27Kip1 occur commonly and are associated with poor prognosis (reviewed in Ref. 10).

Reexpression of p16INK4A, p21Waf1/Cip1, or p27Kip1, expression of dominant negative cdk mutants (11), or introduction of cdk inhibitory peptides (12) in tumor cell lines result in cell cycle arrest or apoptosis or both, depending on the cellular context (5). Therefore, pharmacological agents that modulate cdk activity may be expected to have both cytostatic and cytotoxic effects on tumor growth. E7070 is a synthetic chloroindolyl sulfonamide that targets the G1 phase in a variety of human tumor cell lines (13). Although not a direct inhibitor of cdks, it causes the depletion of cyclin E with a reduction in cdk2 catalytic activity (14). Transcriptional repression of cyclin H also occurs in response to E7070 (15); this reduces cdk7 or cdk-activating kinase activity so that the phosphorylation events required to activate the other cdks do not occur. The reduction in G1 cdk activity induces arrest at the G1-S boundary accompanied by hypophosphorylation of the Rb protein (16). At higher concentrations, E7070 has been associated with up-regulation of p53 and p21Waf1/Cip1, which may also contribute to reduced Rb phosphorylation as well as subsequent apoptosis (16). Treatment with E7070 produces regressions and cures in colorectal cancer and lung cancer xenografts (17).

Toxicities in animals are typical of antiproliferative drugs and include cytopenias and diarrhea. At the maximum-tolerated dose in rats and beagle dogs, sporadic fluctuations of blood glucose and QT interval occurred. In addition, E7070 was shown to inhibit carbonic anhydrase and thus may alter electrolyte balance and intraocular pressure. Phase I studies in man using a variety of schedules have been completed, including a 1-h infusion administered every 3 weeks (18). Dose-limiting toxicities are reversible neutropenia and thrombocytopenia. Other toxicities include anemia, mucositis, hypoglycemia, acneiform rash, phlebitis at the site of injection, and increased INR in patients receiving oral anticoagulation. Pharmacokinetic analyses suggest that this schedule achieves plasma levels comparable with those required to induce cell cycle blockade in preclinical models (18, 19).

This Phase II trial was undertaken to investigate the activity and tolerability of E7070 in patients with incurable SCCHN administered as a 1-h infusion every 3 weeks. In addition, we sought to confirm the effect of E7070 on cdk activity in tumor fine needle aspirates obtained before and after treatment by immunohistochemistry for Rb and its phosphorylated forms.

PATIENTS AND METHODS

Eligibility. Enrolled patients had histologically confirmed SCCHN that was either metastatic at presentation or had failed to respond to or relapsed from first or second line treatment, including surgery, radiation therapy, induction chemotherapy, chemoradiation, or a maximum of one regimen of chemotherapy for recurrent disease. Patients could not have had chemotherapy, radiotherapy, or investigational treatment within 4 weeks of study entry and were required to have measurable disease according to Response Criteria in Solid Tumors (20).

Patients had to be ≥18 years old, with a Karnofsky performance status ≥70% and a life expectancy of at least 4 months, with adequate hematological parameters (hemoglobin > 9 g/dl, neutrophils > 1.5 x 10^9/liter, and platelets > 100 x 10^9/liter), renal sufficiency (serum creatinine < 1.5 x upper limit of normal or creatinine clearance ≥ 60 ml/min), and hepatic function (serum bilirubin < 1.5 mg/dl, alanine transaminase or aspartate transaminase < 2.5 x upper limit of normal). Patients with central nervous system metastases, uncontrolled infection, cardiac dysfunction, or known hypersensitivity to sulfonamides were excluded. Written informed consent was required. The protocol was approved by the institutional review boards of the participating centers.

Treatment Plan. Medical history, physical examination, Karnofsky performance status, biochemical profile, and complete blood count were performed within 14 days before starting treatment. Eligible patients received E7070 at 700 mg/m² as a single 1-h i.v. infusion at 21-day intervals. E7070 was reconstituted in 500 ml of 0.9% NaCl. If local reactions, including irritation, pain, or phlebitis, occurred, the volume was increased to 1000 ml. Biochemical profile and complete blood count were repeated weekly. Treatment delays of up to 2 weeks were permitted for adequate hematological recovery. Up to two dose reductions of 25% were permitted and mandated for grade 4 or complicated grade 3 hematological toxicity or other drug-related grade 3 toxicities that could not be managed with appropriate supportive care. Toxicity was evaluated weekly using the National Cancer Institute Common Toxicity Criteria, version 2. Because E7070 inhibits carbonic anhydrase, intraocular pressure was measured during screening and immediately after the first dose. Tumor assessment was performed clinically before each cycle and with imaging studies after alternate courses. Response was assessed using Response Criteria in Solid Tumors criteria. Treatment was continued until disease progression or unacceptable toxicity occurred.

Fine Needle Aspiration and Tissue Preparation. Tumor fine needle aspiration was performed in consenting patients before and within 24 h of completion of the first infusion of E7070. The decision to perform posttreatment tumor sampling on day 2 was made after observations of G1-S arrest accompanied by apoptosis at this time point in endobronchial non-small cell lung cancers in E7070-treated patients (21) and because of the reported 28-h half-life of E7070 with this dose and schedule (18). Three to five passes were performed on superficial palpable lesions known to be malignant using a 23-gauge needle. The cellular material was rinsed in RPMI medium, centrifuged to form a cell pellet, and fixed in formalin for embedding in paraffin.

Immunohistochemistry. Formalin-fixed, paraffin-embedded 5-µM sections were mounted on glass slides, deparaffinized, and rehydrated through graded alcohols. Immunohistochemistry was performed by an automated stainer (BioGenex, San Ramon, CA). For antigen retrieval, sections were subjected to microwaving in 10 mM citrate buffer (pH 6.0) in a 750-W oven for 15 min. Steps
performed by the autostainer included blocking with hydrogen peroxide and protein, incubation with primary antibody, application of a secondary antibody conjugated to the avidin-biotin peroxidase complex and visualization with 3',3'-diaminobenzidine as a substrate with standard development times (22). Counterstaining was performed with Mayer’s hematoxylin.

Antibodies used for assessment included anti-Rb clone G3-245 (1:200; PharMingen, San Diego, CA), recognizing hyper- and hypophosphorylated forms and the phospho-specific antibodies anti-Rb (pT821) (1:200; Biosource International, Camarillo, CA), representing a site phosphorylated by cdk2; anti-Rb (pS795) (1:400; Cell Signaling Technology, Bedford, MA), representing a site phosphorylated by both cdk2 and cdk4; and anti-Rb (pS807/811) (1:50; Cell Signaling Technology), representing a site phosphorylated by cdk4 (23–25). Samples were also stained with anti-Ki67 and anti-PCNA antibodies, as well as for terminal deoxynucleotidyl transferase-mediated nick end labeling analysis (26).

These antibodies were first characterized for their ability to detect reduced amount of phosphorylated Rb in SCCHN cell lines treated with E7070. For this work, SCC9 and SCC15 cells, obtained from the American Type Culture Collection and grown in RPMI supplemented with 10% FBS, were treated with DMSO or 25, 50, or 100 μg/ml E7070 for 24 h. To analyze the effect of E7070 on cell cycle progression, treated cells were fixed in ethanol, treated with RNase A, stained with propidium iodide, and analyzed for DNA content by flow cytometry using the ModFit program (Verity Software House, Topsham, ME). Alternatively, nuclear lysates were prepared using NE-PER Nuclear Extraction reagents (Pierce, Rockford, IL) and subjected to Western blotting using standard procedures. Parallel plates of treated cells were released by trypsinization, pelleted by centrifugation, fixed for embedding in paraffin, and used for the preparation of slides in a procedure identical to the handling of fine needle aspirates obtained from patients. Immunohistochemical staining of SCC9 cells was used to determine optimal antibody dilutions. Antibody specificity was confirmed by immunohistochemical staining using appropriate control peptides, as well as by lack of nuclear staining in the Rb-negative osteosarcoma cell line SAOS-2.

Approximately 200 cells from each cell block, derived from either primary tumor or a cell line, were scored for Rb staining from 0 to 3+ on the basis of intensity. The number staining 2 or 3+ was expressed as a percentage. Scoring for Ki-67 and proliferating cell nuclear antigen (PCNA) was based on the percentage of positive cells. Histological examination was performed on all samples by two cytologists, who were blinded to the timing of the fine needle aspiration.

**Statistical Analysis.** Because E7070 is felt to act primarily as a cytostatic agent, the primary objective of this open-label Phase II study was to measure progression-free survival at 4 after treatment, measured from the first day of treatment to the first date of either documented progression or death, overall survival, tolerability of E7070, and confirmation of dephosphorylation of the Rb protein in tumor cells after treatment. All patients who were enrolled and received drug were included in the toxicity and efficacy analyses.

The trial used a two-stage design, requiring enrollment of 15 patients during the first stage and an additional 25 patients in the second stage. If at the end of the first stage no patient achieved progression free survival of ≥4 months, the trial would be stopped. The expected improvement in progression-free survival was 2 months when compared with a historical control of 83 patients treated with cisplatin monotherapy with progression-free survival of 2 months (27). This design provided an α level of 5% and a power of 80%. Median progression-free and overall survival were estimated by the method of Kaplan and Meier (28).

**RESULTS**

**Patient Characteristics.** Fifteen patients were treated with E7070 between May 2001 and September 2002 (Table 1). All patients had previously received radiation therapy. Twelve patients had received prior chemotherapy, 5 patients had received one prior regimen, and 7 patients had received two prior regimens. The majority of patients had tumors of oropharyngeal origin (11 of 15) and 4 of 15 had lung metastases. The median age of the enrolled population was 59 years (range, 49–73 years).

**Toxicity.** Two patients withdrew because of adverse events. One patient, who had stable disease through 4 cycles, was hospitalized 10 days after the administration of cycle 5 for syncope, hypotension, and grade 4 anemia and subsequent grade 3 thrombocytopenia. Despite correction of anemia and thrombocytopenia, on day 23, he developed profuse bleeding from an area of tumor ulceration on his neck, requiring placement of a tracheostomy and ventilatory as well as pressor support. Recurrent hemorrhage from the tracheostomy site and from the necrotic tumor precipitated his death on day 39. A second patient developed fever, pancytopenia, pneumonia, and respiratory failure 7 days after the first administration of E7070. She recovered and was removed from the study. There were no other cases of febrile neutropenia. Two patients developed grade 3/4 anemia (13%), grade 4 neutropenia (13%), and grade 3/4 thrombocytopenia (13%). No QTc changes were observed. One pa-

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Patient characteristics (n = 15)</th>
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<tbody>
<tr>
<td>Characteristic</td>
<td>No. of patients</td>
</tr>
<tr>
<td>Age, median 59 years (range 49–73 years)</td>
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</tr>
<tr>
<td>Race/Ethnicity</td>
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<tr>
<td>Caucasian</td>
<td>13 (86%)</td>
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<tr>
<td>African American</td>
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<tr>
<td>Hispanic</td>
<td>1 (7%)</td>
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<tr>
<td>Sex</td>
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</tr>
<tr>
<td>Male</td>
<td>12 (80%)</td>
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<tr>
<td>Female</td>
<td>3 (20%)</td>
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<tr>
<td>Tumor primary</td>
<td></td>
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<tr>
<td>Oropharynx</td>
<td>11 (73%)</td>
</tr>
<tr>
<td>Nasopharynx</td>
<td>4 (27%)</td>
</tr>
<tr>
<td>Prior treatment</td>
<td></td>
</tr>
<tr>
<td>Surgery</td>
<td>15 (100%)</td>
</tr>
<tr>
<td>Radiation therapy</td>
<td>15 (100%)</td>
</tr>
<tr>
<td>Chemotherapy</td>
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</tr>
<tr>
<td>Prior chemotherapy</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>3 (20%)</td>
</tr>
<tr>
<td>One regimen</td>
<td>5 (33%)</td>
</tr>
<tr>
<td>Two regimens</td>
<td>7 (47%)</td>
</tr>
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</table>
tient demonstrated U wave changes on an electrocardiogram after treatment, attributed to hypokalemia. Grade 3/4 biochemical abnormalities are shown in Table 2. Five patients reported an infusion site reaction manifesting as grade 1 pain or, in one case, as a local extravasation considered grade 2 in severity. In all cases, E7070 was subsequently administered uneventfully in 1000 ml. There were no significant changes in intraocular pressure after treatment with E7070. No patient underwent dose reduction or treatment delay.

Efficacy. Thirty-nine cycles of E7070 were administered, with a median of 2.6 cycles/patient (range, 1–5 cycles). Six patients had stable disease after 2 cycles and went on to receive 1 (2 patients), 2 (2 patients), and 3 additional cycles (2 patients), respectively. One of these patients ultimately experienced hemorrhage from a necrotic tumor site, and treatment effect versus progressive disease could not be distinguished. The remainder were removed for progressive disease. Of the other 9 patients, 6 patients had progressive disease after 2 cycles. Three patients were removed after 1 cycle, 2 patients for progressive disease, and 1 patient following recovery from febrile neutropenia and respiratory failure. None of the patients achieved a partial or complete response. Of the 4 patients (27%) free of progression for 3 months, one patient, who had not received prior chemotherapy, had a reduction in tumor volume of 12% after four courses of E7070. However, because no patient achieved a progression-free survival of 4 months, the study was terminated after 15 patients were treated based on the early stopping rule. The median progression free survival was 43 days (95% confidence interval 42, 83 days), and the median overall survival was 8.6 months (95% confidence interval 4.7, 11.2 months).

Modulation of Rb Phosphorylation by E7070 in vitro and in vivo. To characterize available phospho-specific anti-Rb antibodies, SCCHN cell lines were treated with vehicle (DMSO) or E7070 at concentrations ranging from 25 to 100 μg/ml. These concentrations are comparable with those achieved in patients’ plasma. For example, in the Phase I cohort of patients receiving E7070 at 700 mg/m² once every 3 weeks, the Cmax was 80.52 ±/− 22.57 μg/ml, and plasma concentrations remained ≥20 μg/ml for at least 48 h (18). In SCC9 and SSC15 cells, E7070 achieved potent and dose-dependent G1 arrest (Fig. 1A). Over a 24-h exposure, only minimal cytotoxicity occurred, as evidenced by appearance of a sub-G1 peak representing <10% of the cellular DNA content at the highest concentration (data not shown). Western blotting of nuclear lysates demonstrated a reduction in phosphorylation at most known cdk2- and cdk4-phosphorylation specific sites (Fig. 1B). To ensure that these antibodies were adaptable for immunohistochemistry, treated SCC9 cells were removed from the plate by trypsinization, pelleted, fixed in formalin, and embedded in paraffin. Sections mounted on slides were stained, demonstrating a dose-dependent reduction in nuclear phospho-Rb staining, while staining of total Rb was preserved (Fig. 2 and Table 3).

Five patients enrolled at the Dana-Farber Cancer Institute consented to pre- and posttreatment tumor fine needle aspiration. In one patient, adequate cellular material was not obtained from the pretreatment aspirate. In a second patient, clusters of tumor cells in both the pre- and posttreatment aspirates were negative for Rb staining with all antibodies, suggesting that this patient harbored an Rb-negative tumor. Aspirates from the other 3 patients were informative. In all patients, there was a reduction in the percentage of tumor nuclei staining 2+ or 3+ with the anti-Rb (pT821) and (pS795) antibodies; in the third patient, adequate material was available for additional analyses and a reduction in the percentage of tumor nuclei staining strongly with the anti-Rb (pS807/811) was also seen. In 2 of these patients, we were able to document that the decreases in phosphorylated Rb occurred while staining for total Rb was largely preserved (Fig. 3 and Table 4).

Reduced phosphorylation of Rb posttreatment did not correlate with durability of stable disease; the 3 patients from whom the tumor was analyzed had progressive disease after 2, 4, and 5 cycles of treatment, respectively. Exposure to E7070 was not associated with induction of apoptosis, and terminal deoxynucleotidyl transferase-mediated nick end labeling staining of tumor cells was weak both before and after treatment (data not shown). The three tumors analyzed all had low indices.

### Table 2  Grade 3/4 biochemical abnormalities

<table>
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<tr>
<th>Biochemical abnormality</th>
<th>No. of patients</th>
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<tbody>
<tr>
<td>Hypokalemia</td>
<td>3 (20%)</td>
</tr>
<tr>
<td>Hypophosphatemia</td>
<td>1 (7%)</td>
</tr>
<tr>
<td>Hypoglycemia</td>
<td>1 (7%)</td>
</tr>
<tr>
<td>Hypoalbuminemia</td>
<td>1 (7%)</td>
</tr>
<tr>
<td>Hypocalcemia</td>
<td>1 (7%)</td>
</tr>
</tbody>
</table>

Fig. 1. A. SCC9 cells were treated with DMSO or E7070 for 24 h, and DNA content was analyzed by flow cytometry. B, SCC9 and SSC15 cells were treated with DMSO (D) or E7070 at the indicated concentrations; nuclear lysates were analyzed for phospho-retinoblastoma (Rb) and total Rb with the indicated antibodies.
of proliferation by Ki67 staining. In two tumors, the reduction in Rb phosphorylation was accompanied by reduced staining for either Ki67 or PCNA (Table 4).

**DISCUSSION**

No cytotoxic antitumor activity of E7070 was observed in patients with advanced SCCHN with the dose and schedule used in this trial. Among the 15 patients enrolled, 4 patients had stable disease ranging from 12 to 15 weeks, although none were progression free at 16 weeks so that the early stopping rule was invoked. The median time to progression of 43 days and the overall survival of 8.6 months are comparable with results with second-line single-agent chemotherapy regimens and suggest only limited clinical use. Grade 3 and 4 events were uncommon and confirmed the dose-limiting hematological toxicity of E7070 observed in Phase I trials.

The disruption of cell cycle control in human cancer has made the cdk s attractive anticancer drug targets. To date, only a limited number of compounds with cdk inhibitory activity have entered clinical trial, including flavopiridol, UCN-01, roscovitine, and BMS-387032 (29, 30). Although only occasional responses in single-agent chemotherapy regimens and suggest only limited clinical use. Grade 3 and 4 events were uncommon and confirmed the dose-limiting hematological toxicity of E7070 observed in Phase I trials.

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Unlike these compounds, E7070 does not competitively inhibit the ATP binding site of the cdk enzymes. Although its molecular target is not yet known, it modulates cell cycle progression indirectly, resulting in down-regulation of G₁ and G₂ cdk activity and inducing cellular effects similar to those caused by direct cdk inhibitors (14, 15).

A lack of pharmacodynamic end points has complicated trials in which cdk inhibition is the therapeutic goal. For E7070, this is especially important because the drug has recently been reported to be highly protein bound so that pharmacokinetic parameters may not be predictive of successful target inhibition (31). The cell cycle events modulated by E7070 result in reduced phosphorylation of the Rb protein, a critical cdk substrate during the G₁, S-phase transition. In this work, phospho-specific anti-Rb antibodies recognizing cdk4- and cdk2-phosphorylated sites were used to demonstrate reduced Rb phosphorylation after treatment and, by inference, inhibition of cdk s responsible for those phosphorylation events. In 2 of the 3 patients for whom informative material was available, expression of total Rb was preserved, assayed with an antibody recognizing both phospho- and unphosphorylated forms. Of note, Rb staining was not detected in tumor aspirates from 1 of the 5 patients who underwent sampling, consistent with previous studies indicating that ~20% of SCCHN is Rb-negative (32).

Because this was a single-agent study, it is likely that the dephosphorylation of Rb was related to the modulation of cdk activity by E7070. However, in combination therapy trials, Rb dephosphorylation may not be the most appropriate pharmacodynamic marker for E7070 because it is possible that effects on Rb could also be seen with other growth inhibitory agents.

### Table 3: Immunohistochemical scoring of control and E7070-treated SCC9 cells

<table>
<thead>
<tr>
<th></th>
<th>T821</th>
<th></th>
<th>S795</th>
<th></th>
<th>S807/811</th>
<th>Total Rb</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>2, 3+</td>
<td>1+</td>
<td>0</td>
<td>2, 3+</td>
<td>1+</td>
<td>0</td>
</tr>
<tr>
<td>DMSO</td>
<td>34</td>
<td>35</td>
<td>31</td>
<td>67</td>
<td>26</td>
<td>6</td>
</tr>
<tr>
<td>E7070-50</td>
<td>3</td>
<td>21</td>
<td>76</td>
<td>62</td>
<td>14</td>
<td>23</td>
</tr>
<tr>
<td>E7070-100</td>
<td>3</td>
<td>16</td>
<td>81</td>
<td>30</td>
<td>44</td>
<td>26</td>
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</table>

*a Rb, retinoblastoma.*
including DNA-damaging agents that can induce p21\textsuperscript{Waf1/Cip1} in cells expressing wild-type p53. On the other hand, in recent \textit{in vitro} studies of gemcitabine or hydroxyurea followed by flavopiridol, no effects on Rb were observed when the antimetabolites were used alone, and reduced phosphorylation of cdk targets only occurred in the presence of flavopiridol (33, 34). Nonetheless, as pharmacodynamic end points for cdk modulatory drugs are developed, it will be important to also assess phosphorylation of other targets such as p27\textsuperscript{kip1} (35), E2F-1 (36), p220(NPAT) (37), and survivin (38).

Reduction of Rb phosphorylation was not associated with induction of apoptosis because the small percentage of terminal deoxynucleotidyl transferase-mediated nick end labeling-positive cells did not increase in posttreatment aspirates. Evidence of decreased tumor proliferation was obtained in posttreatment samples from 2 patients. However, we did not observe a consistent reduction in proliferation, as indicated by persistent Ki67 staining in posttreatment samples from patient 2. These results must be interpreted with caution because the overall proliferative rates of all analyzed tumors were low at outset. Moreover, correlation of Rb dephosphorylation with immunohistochemical indices of tumor proliferation \textit{in vivo} could be complicated by several factors. For example, the long half-lives of Ki-67 and PCNA could affect the persistence of their expression in posttreatment samples (39). In this regard, positive Ki-67 staining has been reported in cell lines even when they are arrested at the

### Table 4

<table>
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<tr>
<th>Patient</th>
<th>Antibody</th>
<th>% (2+, 3+) pretreatment</th>
<th>% (2+, 3+) posttreatment</th>
<th>Clinical outcome</th>
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<tr>
<td>1(^\text{a})</td>
<td>Anti-Rb(^\text{b}(\text{pT821}))</td>
<td>32</td>
<td>5</td>
<td>PD after two cycles</td>
</tr>
<tr>
<td></td>
<td>Anti-Rb (pS795)</td>
<td>86</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>2(^\text{a})</td>
<td>Anti-Rb (pT821)</td>
<td>79</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>3(^\text{a})</td>
<td>Anti-Rb (pT821)</td>
<td>41</td>
<td>0</td>
<td></td>
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<tr>
<td></td>
<td>Anti-Rb (pS795)</td>
<td>80</td>
<td>5</td>
<td>SD after two cycles; PD after four cycles</td>
</tr>
<tr>
<td></td>
<td>Anti-Rb (pS807/811)</td>
<td>80</td>
<td>0</td>
<td>SD after four cycles; PD after five cycles</td>
</tr>
<tr>
<td></td>
<td>Anti-Rb (total)</td>
<td>70</td>
<td>50</td>
<td></td>
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</tbody>
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\(^\text{a}\) Patient 1: Ki67, pretreatment 10%; posttreatment 3%.
\(^\text{b}\) Rb, retinoblastoma; PD, progressive disease; SD, disease.
\(^\text{c}\) Patient 2: Ki67, pretreatment 1%; posttreatment 3%.
\(^\text{d}\) Patient 3: PCNA, pretreatment 30%; posttreatment 20%.
G₁-S boundary using synchronizing inhibitors or by inducing p21(Waf1/Cip1) in a tetracycline-regulated expression system or following DNA damage (39). Furthermore, it is unlikely that synchronous dephosphorylation of Rb at multiple sites in all tumor cells can be achieved in vivo. In some cells, persistence of phosphorylation at a few cdk sites in a subset of Rb molecules may permit continued entrance of some cells into S phase. In other cells, Rb dephosphorylation may occur during the G₁-S phases, which could slow cell cycle progression but may not result in reduction of Ki-67 and PCNA expression (40, 41).

Although the data suggest inhibition of cdk activity with a concomitant decrease in cellular proliferation, these effects were most likely transient. In the absence of apoptosis, it is likely that tumor cells recovered once the drug was metabolized, permitting continued Rb phosphorylation and cell cycle progression. In this regard, the use of a primarily cytostatic agent at 3-week intervals may not be optimal. Four Phase I trials of E7070 have shown that this regimen results in a presecond dose agent with potent antitumour activity in vivo and in vivo. Eur J Cancer 2001;37:2275–2282.

REFERENCES

27. Jacobs C, Lyman G, Velzer-Garcia E. A Phase III randomized study comparing cisplatin and fluorouracil as single agents and in combination...
A Phase II Clinical and Pharmacodynamic Study of E7070 in Patients with Metastatic, Recurrent, or Refractory Squamous Cell Carcinoma of the Head and Neck: Modulation of Retinoblastoma Protein Phosphorylation by a Novel Chloroindolyl Sulfonamide Cell Cycle Inhibitor


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