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Thomas Decker, Michael Sandherr, Katharina Goetze, Madlen Oelsner, Ingo Ringshausen, et al.. A pilot trial of the mTOR (mammalian target of rapamycin) inhibitor RAD001 in patients with advanced B-CLL. *Annals of Hematology*, 2008, 88 (3), pp.221-227. 10.1007/s00277-008-0582-9 . hal-00477973

**HAL Id: hal-00477973**

**<https://hal.science/hal-00477973>**

Submitted on 30 Apr 2010

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# A pilot trial of the mTOR (mammalian target of rapamycin) inhibitor RAD001 in patients with advanced B-CLL

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Received: 27 February 2008 / Accepted: 24 July 2008 / Published online: 14 August 2008  
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**Abstract** Although B-cell chronic lymphocytic leukemia (CLL) is treatable, it remains an incurable disease and most patients inevitably suffer relapse. Many therapeutic options exist for those requiring therapy, including monoclonal antibodies and stem cell transplantation, but remissions tend to last shorter in the course of the disease. Targeting the cell cycle has recently been realized to be an attractive therapeutic approach in solid and hematological malignancies, and the proliferative nature of B-CLL is increasingly accepted. Here, we report data on a phase II pilot trial with the oral mammalian target of rapamycin (mTOR) inhibitor RAD001 5 mg/daily in patients with advanced B-CLL who had progressive disease after at least two lines of treatment. After treatment of seven patients, this trial was stopped because of toxicity concerns, although some degree of activity was observed (one partial remission, three patients with stable disease). Interestingly, cyclin E expression decreased

in responding patients. Further strategies of mTOR inhibition by RAD001 in B-CLL should focus on different treatment schedules, adequate anti-infectious prophylaxis, or combinations with cytotoxic drugs.

**Keywords** CLL · MTOR · RAD001

## Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in Western countries and is characterized by the progressive accumulation of nondividing small malignant B-cells that are arrested in the G<sub>0</sub> phase of the cell cycle [1]. For decades, B-CLL was regarded as a prototype disease caused by the slow accumulation of cells defective in apoptosis with an imbalance of expressed bcl-2 family proteins [2]. However, it seems highly unlikely that disease progression can be explained only by the existence of mature malignant B cells incapable of cell death or cell cycle progression. The proliferative nature of B-CLL has been realized only recently [3], and disease progression seems to rely upon cycling B-CLL cells in proliferation centers in central lymph organs [4, 5]. Therefore, targeting the proliferating pool of cells seems to be an attractive therapeutic option [6].

We have previously shown that rapamycin or its analogon RAD001 blocks cell cycle progression in CLL cells by interfering with expression of critical cell cycle molecules, suggesting that mammalian target of rapamycin (mTOR) represents an attractive target for therapy of CLL [7, 8]. Furthermore, rapamycin was able to delay disease progression in mice with a lymphoproliferative disease [9].

In mammalian cells, mTOR regulates the initiation of translation by activation of eukaryotic initiation factor 4E (eIF4E) and of ribosomal p70 S6 kinase (p70<sup>S6K</sup>). Both are

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T.D., M.S., and K.G. contributed to the clinical care of the patient and interpretation of data. M.O. carried out laboratory investigations and contributed to analysis of data. T.D., M.S., and C.P. designed the study. T.D. wrote the paper.

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This work was supported in part by funding from Novartis Pharma.

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critical steps for the translation of mRNAs that encode proteins essential for G1 cell-cycle progression [10]. Indeed, cell cycle arrest of rapamycin-treated B-CLL cells was accompanied by strongly reduced expression of cyclin D3, cyclin E, and cyclin A [7].

Recently, rapamycin ester-analogs, such as CCI-779 and everolimus (RAD001), with improved stability and solubility have been developed for both oral and intravenous administration. Both drugs have demonstrated antitumor activity in a variety of cell lines [11]. Clinical activity and good tolerability has been demonstrated in solid cancer and hematologic malignancies [12–14]. RAD001 is an orally available macrolide that has been shown to be well tolerated in the setting of solid organ transplantation when it is given daily as part of an immunosuppressant, multidrug regimen consistently including cyclosporin A and glucocorticoids [15].

Therapeutic opportunities have improved dramatically in B-CLL patients, with monoclonal antibodies being incorporated in standard treatment schedules [1, 16] and promising developments in active immunotherapy [17]. However, new therapeutic strategies are clearly needed for patients with fludarabine refractory disease or those patients relapsing after several lines of treatment [18].

Therefore, we planned to conduct a phase II trial with the oral mTOR inhibitor RAD001 in patients with advanced B-CLL cells, relapsing after at least two lines of therapy. Although signs of activity were observed, we stopped the trial because of toxicity concerns. Data on toxicity and clinical response, as well as results of correlative studies regarding cell cycle regulation, are presented.

## Material and methods

### Trial design and patients

This trial was designed as a nonrandomized, one-sample treatment study in phase II of clinical evaluation. A Simon's two-stage optimal design had been adopted to test

the null hypothesis that the probability of progression-free rate at 3 months is <20% vs the alternative >40%. After testing the drug on 18 patients in the first stage, the trial should be terminated if only three or fewer patients remain free from progression at 3 months.

Eligible patients must have a confirmed diagnosis of B-CLL according to clinical and immunophenotypic criteria. Patients must have had at least two different lines of systemic chemotherapy. At least one line of chemotherapy must have included a nucleoside analogon (except for patients with a positive Coombs test). Active disease must be present as defined by the NCI criteria [1]. All patients had a live expectancy of at least 3 months, a performance status of 0 to 2, and adequate hepatic and renal function. Platelets had to be above 30,000/ $\mu$ l and absolute neutrophil counts were required to be >1,000/ $\mu$ l. Patients were not included if they had active hemolytic anemia, central nervous system involvement, congestive heart failure, active symptomatic coronary heart disease, uncontrolled hypertension, or severe pulmonary dysfunction. Seven patients were entered into the trial. All patients were heavily pretreated with two to five lines of therapy. Two patients had not received prior fludarabine treatment because of a positive Coombs test. Patient characteristics are given in Table 1.

### Treatment plan

Patients were treated with 5 mg/day RAD001. Clinical visits were performed weekly during the first 3 months and then every other week and included vital signs, physical examination, complete blood count with differential blood diagnostics and platelet count, and toxicity evaluation.

At staging every 3 months of RAD001 treatment and after discontinuation of RAD001, performance status, height/weight, chest X-ray, lymph node assessment, spleen and liver measurement, sonography of abdomen, and clinical chemistry were performed. Blood counts and clinical visits, RAD001 serum levels, and correlative laboratory studies were done after 4 weeks of treatment and at the

**Table 1** Patients characteristics, outcome, toxicities

Patient	Duration of therapy with RAD001	Binet stage/previous therapies	ZAP-70	Best efficacy outcome	Toxicities
1	7 weeks	C/4	Positive	PD	Pneumonia, thrombocytopenia, mucositis (SAE)
2	9 months	B/3	Positive	SD	EBV Lymphoma, death
3	3 months	C/2	Positive	SD	Thrombocytopenia, mucositis
4	3 months	B/5	Negative	PD	No significant adverse events
5	2 months	B/2	Not done	SD	Pneumonia (PCP) Drowsiness (SAE)
6	4 months	C/3	Positive	PR	Herpes Zoster, Meningitis
7	2 weeks	B/4	Not done	PD	No significant adverse events

discretion of the treating physician. After 3 months of treatment, the addition of prednisolon (100 mg for 4 days) was allowed in cases of leukocyte counts of more than 200,000/ $\mu$ l.

#### Correlative laboratory studies

Peripheral blood samples were drawn before treatment was initiated and after 4 weeks of therapy with RAD001. RAD001 serum levels were determined by a tandem mass spectrometry method as published previously [19]. Separation, cell cycle analysis, and immunoblotting were performed as described previously [7]. An abbreviated summary is provided.

**Separation procedures** Peripheral blood mononuclear cells (PBMNC) were isolated from heparinized blood samples by centrifugation over a Ficoll-Hypaque layer (Biochrom, Berlin, Germany) of 1.077 g/ml density. For separation of CLL B-cells, PBMNC were incubated with anti-CD2 and anti-CD14 magnetic beads (Dynabeads M450, Dynal, Oslo, Norway). Such prepared B cells from CLL patients were >98% pure, as assessed by direct immunofluorescence using a Coulter Epics XL (Coulter, Hamburg, Germany).

**Cell cycle analysis** Approximately  $10^6$  cells were collected and fixed overnight in 70% ethanol at 4°. Cells were then washed and stained with propidium iodide (Sigma, St. Louis, MO, USA) 5  $\mu$ g/ml in the presence of DNase free RNase (Sigma). After 30 min at room temperature, the cells were analyzed via flow cytometry using a Coulter Epics XL cytofluorometer, acquiring 10,000 events.

**Immunoblotting** A total of 1 to  $2 \times 10^7$  cells were lysed as previously described. Lysates were spun at 12,000 rpm for 20 min and supernatant was collected. Protein concentration was assessed by the Bio-Rad assay method (Bio-Rad, Hercules, CA, USA). Total extracts (20  $\mu$ g/lane) were subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotting was performed on PVDF membranes (Immobilon-P, Millipore GmbH, Billerica, MA, USA). Blots were developed using SuperSignal® chemoluminescent substrates from Pierce Chemical Company (KMF GmbH, Rockford, IL, USA). Monoclonal antibodies (Mab) specific for cyclin E (clone HE12) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mabs specific for aktin (clone AC-15) were from Sigma, Deisenhofen, Germany. Mabs specific for p27 (clone G173-524) were purchased from Pharmingen (San Diego, CA, USA). Analysis of ZAP-70 expression was performed with appropriate positive and negative controls as published previously [20] using a ZAP-70 antibody (clone 29) from BD Transduction Laboratory, Mississauga, Canada.

## Results

### Patients and toxicities

Seven patients were enrolled between October 2004 and June 2005 (Table 1). All patients were heavily pretreated, five patients having been treated with fludarabine and one patient being in relapse after autologous stem cell transplantation. Five of seven patients were treated with RAD001 for at least 3 months and treatment appeared to be well tolerated. After 4 weeks of treatment, all patients had RAD001 serum levels of more than 2  $\mu$ g/l (range 2–37.5  $\mu$ g/l)—a concentration well above the concentration needed in vitro for complete G1 arrest [8]. Initially, toxicities were mild and mainly consisted of fatigue, mucositis, and thrombocytopenia—no grade 3 or 4 toxicity occurred.

However, patient 1 developed a severe lobular pneumonia with consecutive and reversible renal failure. A bronchoalveolar lavage was performed, but no definitive microbe was detected. This patient has been heavily pretreated (four lines of treatment including stem cell transplantation). However, the last chemotherapy regimen (fludarabine/cyclophosphamid) was administered more than 1 year before the start of RAD001.

After 3 months of treatment, patient 5 had a confirmed pneumocystitis carinii pneumonia, which he survived after appropriate antibiotic treatment was initiated. This patient has been previously treated with two lines of treatment. The last line of treatment was bendamustin, which was stopped 2 years before initiation of RAD001.

Patient 6 had herpes zoster ophthalmicus with associated meningitis, which led to admission to a stroke unit because of sudden loss of consciousness. The patient recovered after the initiation of aciclovir therapy but severe post zoster neuralgia developed. This patient had been treated previously with three lines of therapy. RAD001 was started because of rapidly progressive disease within 6 months of fludarabine treatment. Therapy was stopped in these three patients due to these severe complications.

In patient 4, therapy with RAD001 was stopped due to rising leukocyte counts after an 8-month period of disease stabilization. Salvage therapy with fludarabine and cyclophosphamid was initiated 4 days after RAD001 therapy was stopped. EBV associated high-grade lymphoma developed within 4 weeks and the patient rapidly died. The clinical course and the possible relationship with RAD001 treatment is discussed elsewhere [21]. Toxicities are presented in Table 1.

### Disease response

Most patients received extensive prior treatment (Table 1). Response was characterized according to the modification

of the National Cancer Working Group Criteria [1]. One partial response was observed in patient 6 after prednisone was added to RAD001 for four consecutive days. When prednisone was added, a mild tumor lysis syndrome accompanied a rapid reduction of leukocyte counts from more than 200,000 to 70,000/ $\mu\text{l}$  within 3 days (Fig. 1). Leukocytes remained stable thereafter but RAD001 had to be stopped after 4 months of treatment because of toxicity (herpes zoster infection as described above). In three patients (patients 2, 3, and 5), RAD001 was able to induce stabilization of leukocyte counts. In two patients (patient 2 and patient 5), time to disease stabilization was longer than 1 month. After 3 months of treatment, patient 3 refused to remain on study due to mucositis and fatigue. Patient 2 had

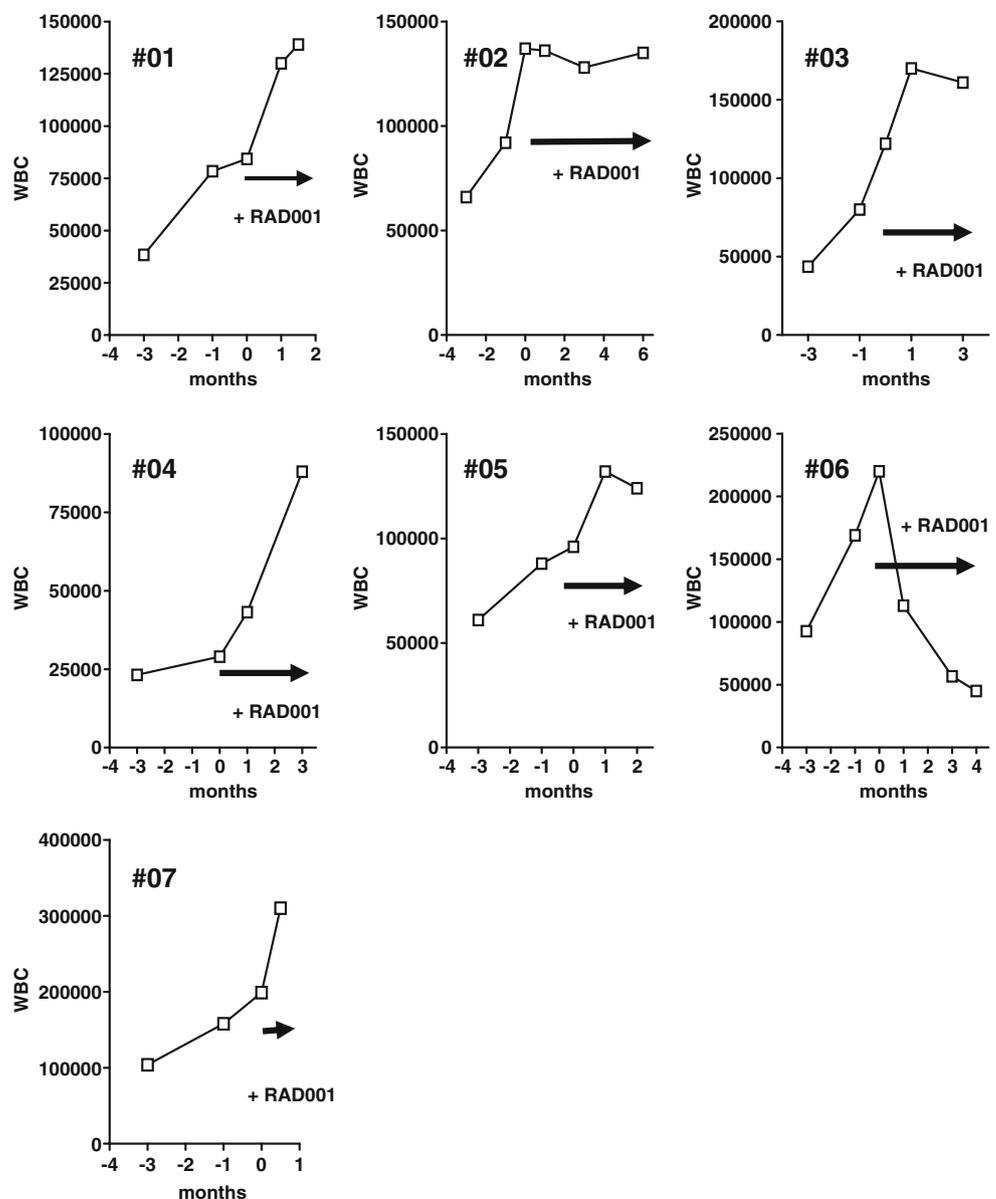
progressive disease after 8 months of therapy and patient 5 was taken off study because of PCP pneumonia.

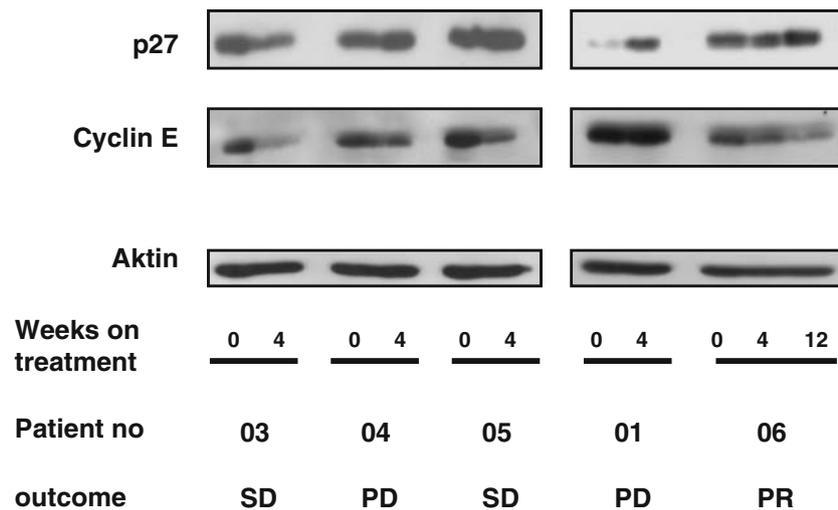
Progressive disease was observed in three patients. In patient 7, high and progressive leukocyte counts left no time to wait for a therapeutic effect. Accordingly, therapy was stopped when leukocyte counts exceeded 300,000/ $\mu\text{l}$ .

#### Correlative studies

Cell cycle analyses were performed in purified B-CLL cells from five patients before and after 4 weeks on treatment with RAD001. As expected, no difference was observed before and after treatment with more than 98% of cells in the G0 phase of the cell cycle in every sample tested (data not shown).

**Fig. 1** Leukocyte counts in B-CLL patients treated with RAD001. Leukocyte counts ( $\mu\text{l}$ ) of seven individual patients treated with RAD001 are depicted. Leukocyte counts were determined 3 months and 1 month before and during treatment with RAD001. The treatment period is indicated by an asterisk





**Fig. 2** Expression of Cyclin E and p27 in B-CLL samples before and after treatment with RAD001. Expression of cell cycle regulatory molecules was analyzed in purified B-CLL cells before and after 4 weeks of treatment with RAD001. Cyclin E, p27, and actin were revealed by immunoblotting with mAb in total cell extracts (50 mg).

We have reported previously that cyclin E is strongly expressed in B-CLL cells and is downregulated upon mTOR inhibition [7, 20, 22]. In addition, the cell cycle inhibitor p27 is also strongly expressed in B-CLL cells and is also regulated in part by mTOR [23, 24]. Therefore, we have analyzed the expression of these cell cycle proteins in highly purified B-CLL cells before treatment with RAD001 and after 4 weeks of treatment. Samples were obtained from five patients. In patient 6 (the one with a partial remission), cells were also obtained after 3 months of treatment. Immunoblots of cyclin E, p27, and aktin are shown in Fig. 2. P27 expression increased in three patients, remained stable in one, and decreased in one sample—no correlation with response to mTOR inhibition could be observed. In contrast, cyclin E expression decreased in all samples, having at least stable disease.

In addition, we analyzed expression and phosphorylation of the mTOR downstream target p70s6 kinase. Consistent with our previous observations, no significant phosphorylation of p70s6 kinase was observed in any sample and expression levels remained unchanged (data not shown).

## Discussion

Inhibition of mTOR is a promising strategy in cancer therapy, and many trials with the Rapamycin analogs CCI-779 and RAD001 are currently underway in different types of cancer. First results of therapy with RAD001 in various hematological malignancies have been recently reported [14]. RAD001 has the advantage of being orally bioavailable. In addition, there are extensive safety data on

In patient 6, an additional analysis after 12 weeks of treatment was performed. Patient number and corresponding best clinical response are provided (*PD* progressive disease, *PR* partial remission, *SD* stable disease)

RAD001 because of the advanced stage of its development in solid organ transplantation. Importantly, no significant infections have been reported when RAD001 was added to the immunosuppressive regimen containing cyclosporine and corticosteroids [25].

Advanced CLL is almost always accompanied by a profound immunosuppression, but even combinations of drugs that cause a profound and long-standing T cell depletion have been reported to be feasible even in heavily pretreated patients [16], although some studies have demonstrated a high rate of infectious complications [26].

However, RAD001 therapy appeared to be accompanied by severe infectious complications in our small series of patients. After the occurrence of four immunosuppression-associated life-threatening complications, we decided to stop the trial in its present form. However, the observed infectious complications could have also been caused by immunosuppressive fludarabine-based therapy finished shortly before (patient 6) or initiated after RAD001 therapy (patient 2).

Antimicrobial prophylaxis was not mandatory in this study but should be incorporated in future trials with mTOR inhibitors in B-CLL. Drugs like acyclovir and cotrimoxazol have well demonstrated their efficacy in preventing pneumocystis carinii- and varicella zoster-associated infections [27]. Severe infections also occurred in the study by Yee et al. when RAD001 was used at a dose of 10 mg/day in patients with hematologic malignancies [14]. Additional toxicities in our trial were mild and included fatigue, thrombocytopenia, and mucositis, comparing favorably with standard cytotoxic treatment.

The aim of this study was to demonstrate the potential of RAD001 to cause disease stabilization. Of seven patients

treated, there was disease stabilization in three patients and a partial response in one patient (Fig. 1). This response rate of (4/7) demonstrates activity of RAD001 in these heavily pretreated patients and would have allowed us to extend the sample size from 18 treated patients in stage I to 35 patients in stage II. However, this trial was stopped because of toxicity concerns. Other new drugs in this setting are also modestly effective and are accompanied by significant toxicities [28, 29]

Interestingly, leukocyte counts were progressive in three of the four responding patients during the first month of treatment with RAD001. Similar findings were described in patients treated with lenalidomide, and such “flare reactions” might mimic disease progression [28]. In fact, in patient 7, who started therapy with more than 200,000 white blood cells/ $\mu$ l, therapy with RAD001 was stopped because of such a flare reaction. Therefore, patients with very high leukocyte counts should not be treated with RAD001.

We have demonstrated previously that mTOR inhibition strongly affects the expression of cell cycle regulatory proteins in activated B-CLL cells [7]. As in our in vitro experiments, cyclin E expression decreased in the samples of patients who had at least disease stabilization upon treatment with RAD001, while no consistent effect on p27 expression was observed. This might point to a therapeutic effect of RAD001 on proliferating cells.

Plasma levels of RAD001 were measured after 4 weeks of treatment in six patients and demonstrated a concentration well above the concentration needed to stop proliferation in vitro [8]. However, it remains uncertain if RAD001 is effective in the proliferation centers in lymph nodes or the bone marrow, where the protective environment might render B-CLL cells unresponsive to treatment with RAD001, as well as cytotoxic drugs [4].

In summary, treatment with the mTOR inhibitor RAD001 is modestly effective in heavily pretreated B-CLL patients with active disease. Future trials with RAD001 should include effective anti-infectious prophylaxis. Given the potency of RAD001 to prevent disease progression rather than induce remissions, maintenance strategies might be tested. Similar concepts are already evaluated with the monoclonal antibody rituximab or other targeted therapies in hematological malignancies [30, 31]. In addition, promising in vitro data have been presented with combinations of RAD001 and chemotherapy, as well as rituximab [11, 32]. Therefore, combination strategies might be more promising in future trials of RAD001 in B-CLL.

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