Original Article
Synergistically suppressive effects on colorectal cancer cells by combination of mTOR inhibitor and glycolysis inhibitor, Oxamate

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Abstract: Colorectal cancer (CRC) is the third most common cancer worldwide, representing a major cancer burden. As a natural mTOR inhibitor, rapamycin has been demonstrated to regulate various cellular biological behaviors of cancer cells, including growth inhibition and induction of apoptosis in multiple types of malignant tumors. In this study, we report mTOR inhibitor treatments significantly decreased colon cancer cells glucose metabolism. The glucose uptake and lactate product of DLD-1 and LoVo cells were suppressed by rapamycin. In addition, rapamycin resistant DLD-1 cells display elevated glycolysis rate. The expressions of glycolysis enzymes, Hexokinase 2, PKM2 and LDHA are upregulated in rapamycin resistant cells. We observed promotion of cellular glycolysis by overexpressing LDHA renders colon cancer cells resistant to rapamycin and inhibition of glycolysis by knockdown LDHA sensitizes colon cancer cells to rapamycin. Importantly, we demonstrate the combination of rapamycin and glycolysis inhibitor, Oxamate showed a synergistically inhibitory effect on colon cancer cells. Our study will contribute to the development of therapeutic approaches through combination of mTOR inhibitor with glycolysis inhibitor for the treatment of colorectal cancer patients.

Keywords: Colorectal cancer, mTOR inhibitor, glycolysis inhibitor, Oxamate

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide [1]. Chemotherapy has been used for the treatment of metastatic colorectal cancer [2]. In most cases, colon cancers are curable by combination of surgical excision and chemotherapy [3]. However, once tumors metastasize to distant sites, they become difficult to be excised, with relative low 5-year relative survival rate (around 12%) [4].

The serine/threonine kinase mTOR is import in the phosphoinositide 3-kinases (PI3K) signaling pathway, which regulates the cancer cell growth and metabolism [5], chemosensitivity [6], metastasis and invasion [7] through upregulating the translation, lipid synthesis, and metabolism. mTOR exists in two multiprotein complexes: mTORC1 and mTORC2, which have distinct regulatory subunits and specific substrates [8]. mTORC1 is rapamycin-sensitive and

is essential for the growth and proliferation of cancer cells [8]. It has been widely studied that mTOR inhibitors are effective anti-tumor agents for treating and preventing CRCs tumor progression [9-12]. Therefore, developing potential and effective mTOR inhibitors against CRCs is needed for benefiting tumor patients.

Cancer cells exhibit different characteristics from normal cells that they preferentially metabolize glucose through anaerobic glycolysis, even in the presence of abundant oxygen [13]. This phenomenon is called "Warburg effect". Recently, combined treatment with classical chemotherapeutic agents with glycolysis inhibitors has been proposed to be an effective approach against tumors [14]. The objective of this study was to determine the effective of mTOR inhibition on the cellular glucose metabolism and assess the susceptibility of colorectal cancer cell lines to rapamycin and glycolysis inhibitor, Oxamate. This study may contribute to

the development of therapeutic agents through combination of mTOR inhibitor with glycolysis inhibitor for the treatment of colorectal cancer patients.

Materials and methods

Cell lines and cell culture

The human colorectal cancer cell lines DLD-1 and LoVo were purchased from the American Type Culture Collection (ATCC) and cultured according to the conditions recommended by ATCC. DLD-1 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS). LoVo cells were cultured in DMEM/F12 medium containing 10% FBS. All cells were cultured at 37°C with 5% CO_2. Rapamycin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethylsulfoxide (DMSO).

Plasmid DNA and siRNA transfection

Cells were transfected with control vector or LDHA overexpression vector at 2 μg in a 6-cm dish for 72 hours. Cells were transfected with control siRNA or siLDHA at 50 nM for 72 hours. All transfection was performed using the Lipofectamine 2000 transfection reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions.

Cell viability assay

Cell viabilities were measured by MTT assay. Briefly, cells were plated in a 96-well plate at a density of $5×10^3$ cells per well for overnight. MTT (Promega, Madison, WI, USA) was added to the medium of cells with drugs or control treatments at a final concentration of 0.5 μg/ml. Cells were incubated at 37°C with 5% CO_2 for 3 hours. Medium was then removed and 100 μl DMSO was added to each well. After gently rotated on an orbital shaker for 10 min, plate was put into a microplate reader (Bio-Rad, Hercules, CA, USA) and the absorbance was detected at 570 nm. All experiments were performed in triplicate.

Real-time quantitative PCR (qRT-PCR)

Total RNA was extracted from cells using the RNeasy RNA isolation kit (Qiagen, Hilden, Germany). The quality of RNA was checked then treated with DNase I (0.4 units/μg RNA). RNA samples were reverse transcribed into cDNA using the miScript II RT kit (Qiagen, Hilden, Germany). cDNA was then amplified by real-time PCR using SYBR-Green dye Universal Master mix using the Applied Biosystems 7300 system (Applied Biosystems, Foster City, CA, USA). Primers of the glycolysis genes in this study were: HK2: forward, 5'-CAAAGTGAC-AGTGGGTGTGG-3' and reverse, 5'-GCCAGGTCC-TTCACTGTCTC-3'; PKM2: forward, 5'-AGAACTT-GTGCGAGCCTCAA-3' and reverse, 5'-GAGCAG-ACCTGCCAGACTC-3'; LDHA: forward, 5'-GGAT-GAGCTTGCCCTTGTTGA-3' and reverse, 5'-GAC-CAGCTTGGAGTTCGCAGTTA-3'; GAPDH forward, 5'-CTCTGCTCCTCCTGTTCGAC-3' and reverse, 5'-ACCAAATCCGTTGACTCCGA-3'. The PCR conditions included an initial holding period at 95°C for 5 min, followed by a two-step PCR program consisting of 95°C for 5 sec and 60°C for 30 sec for 45 cycles. All samples were normalized against the internal control (GAPDH) and analyzed using the $2^{-\Delta\Delta Ct}$ method.

Measurement of glycolytic activity

The cellular glycolytic activity was determined by measuring the glucose uptake and lactate production. The glucose uptake was measured using the Glucose Uptake Colorimetric Assay Kit (K676, BioVision, Mountain View, CA, USA). The lactate concentration in the culture medium was measured using the Lactate Colorimetric Assay Kit II (K627, BioVision, Mountain View, CA, USA). All assays were performed according to the manufacturer's instructions. Results were normalized by protein concentrations of each samples and experiments were performed in triplicate.

Western blotting

Total proteins from cells were extracted by radioimmunoprecipitation assay (RIPA) lysis buffer. Protein concentration was determined by Bradfod assay. Equal amount protein of each samples was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following SDS-PAGE, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked in 5% non-fat dried milk in TBST for 1 hour at room temperature and then incubated with specific primary antibodies for overnight at 4°C. Antibodies used in this study: LDHA, PKM2
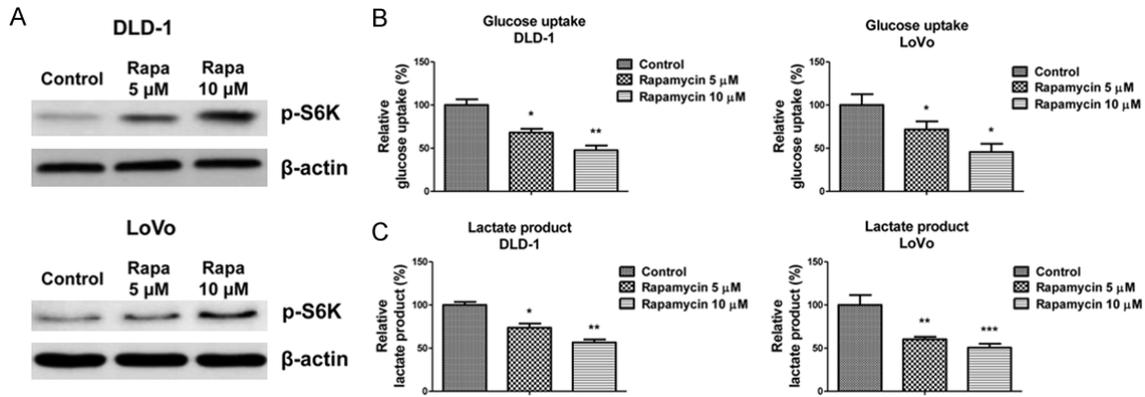
Figure 1. Rapamycin suppresses glucose metabolism of colon cancer cells. (A) DLD-1 (upper) and LoVo (lower) cells were treated with DMSO (control) or with rapamycin at 5 or 10 μM for 48 h. Phosphorylation of S6K was measured by Western blot. β-actin was a loading control. (B) DLD-1 cells and (C) LoVo cells were treated with DMSO (control) or with rapamycin at 5 or 10 μM for 48 h. The glucose (left) and lactate product (right) were measured. *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$, compared with the control.

and Hexokinase 2 were purchased from Cell Signaling Technology (Glycolysis Antibody Sampler Kit #8337, Danvers, MA, USA); β-actin was purchased from Santa Cruz (#sc-47778, Dallas, TX, USA). After washing, membranes were incubated with the second antibody (Cell Signaling Technology, Danvers, MA, USA) for 1 hour at room temperature; immune complexes were detected using the enhanced chemiluminescence (ECL) method. Experiments were performed in triplicate.

Statistical analysis

Statistical analysis was performed using Prism 5.0 statistical software. Data were expressed as the mean ± standard deviation (SD) of triplicate experiments and analyzed by one-way analysis of variance (ANOVA) or t-test for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Inhibition of mTOR suppresses glycolysis of colon cancer cells

Reprogramming of cellular metabolism is an important characteristic of cancer cells [15]. In this study, to determine the regulatory effects between mTOR signaling pathways and dysregulated glucose metabolism, we treated two colorectal cancer cell lines DLD-1 and LoVo with rapamycin at multiple concentrations. As we expected, treatments of colon cancer cells by rapamycin significantly suppressed mTOR pa-

thway (**Figure 1A**). The phosphorylation of S6K was downregulated by rapamycin treatments at 5 or 10 μM. Interestingly, we observed the glucose uptake (**Figure 1B**) and lactate product (**Figure 1C**) were suppressed by rapamycin treatments, suggesting targeting dysregulated glucose metabolism might contribute to the anti-cancer effects of mTOR inhibitors on colon cancer cells.

Rapamycin resistant colon cancer cells display elevated glycolysis

To investigate whether the dysregulated glucose metabolism of colon cancer cells correlates with rapamycin sensitivity, we treated DLD-1 cells with gradually increased concentrations of rapamycin to generate the rapamycin resistant cells. Results in **Figure 2A** showed rapamycin resistant DLD-1 cells could tolerate higher concentrations of rapamycin treatments. The IC50 to rapamycin is 23.43 μM in DLD-1 parental cells, and the IC50 to rapamycin increases to 52.47 μM in DLD-1 rapamycin resistant cells (**Figure 2A**). Consistently, caspase-3 activities in DLD-1 cells were induced by rapamycin treatments; however, rapamycin resistant DLD-1 cells did not showed alterations of caspase-3 activity under rapamycin treatments (**Figure 2B**). We next measured the glucose metabolism and found both glucose uptake (**Figure 2C** upper) and lactate product (**Figure 2C** lower) were significantly upregulated in DLD-1 rapamycin resistant cells compared with sensitive cells. Consistently, the glycolysis enzymes Hexokinase 2 (HK2), LDHA, and PKM2
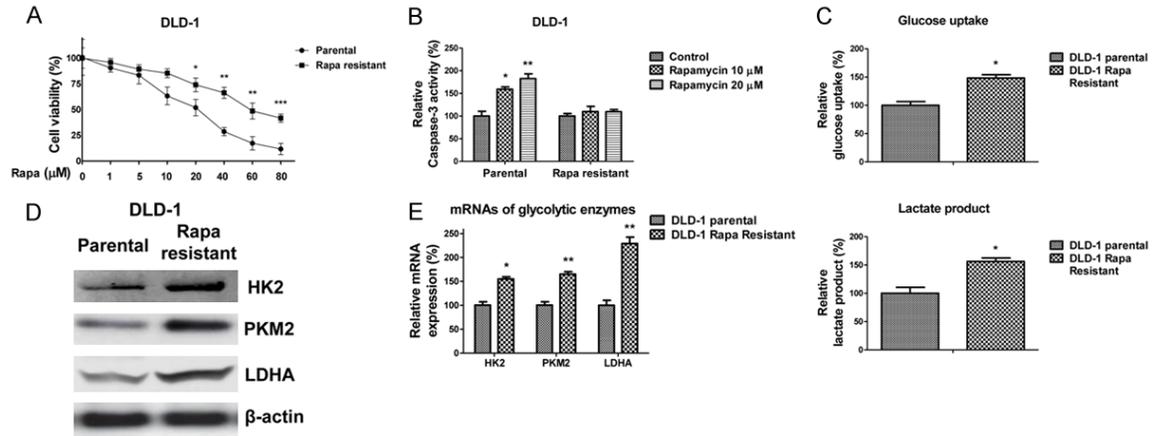
Figure 2. Rapamycin resistant colon cancer cells display increased glucose metabolism. A. DLD-1 parental or resistant cells were treated with rapamycin at 0, 1, 5, 10, 20, 40, 60 or 80 µM for 48 h, cell viability was then determined by an MTT assay. B. DLD-1 parental or resistant cells were treated with rapamycin at 0, 10 or 20 µM for 48 h, activities of Caspase-3 were measured. C. Glucose uptake (upper) and lactate product (lower) were measured in DLD-1 parental or rapamycin resistant cells. D. Expressions of HK2, PKM2 and LDHA were measured from DLD-1 parental or rapamycin resistant cells by Western blot. β-actin was a loading control. E. mRNAs of HK2, PKM2 and LDHA were measured from DLD-1 parental or rapamycin resistant cells by qRT-PCR. *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$, compared with the control.
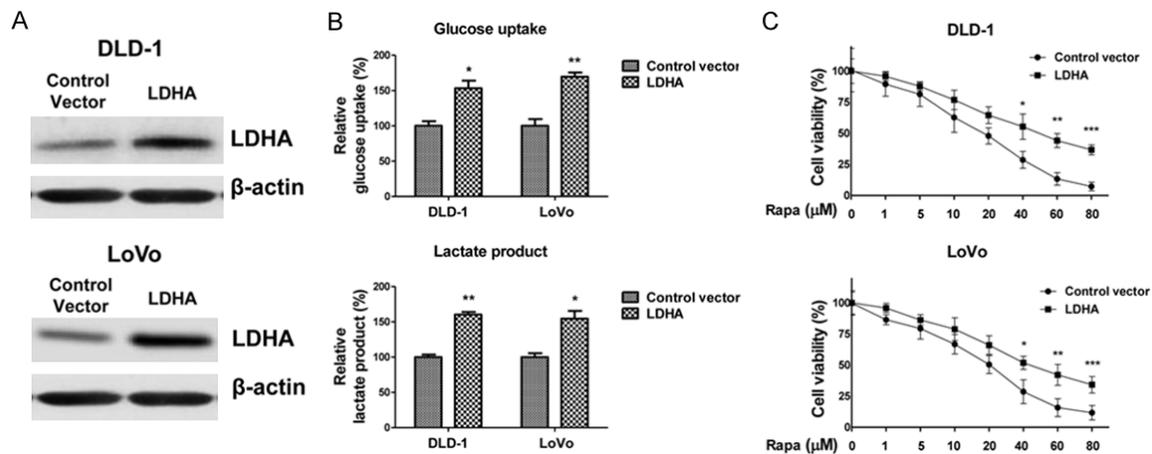
Figure 3. Overexpression of LDHA renders colon cancer cells resistant to rapamycin. A. DLD-1 (upper) and LoVo (lower) cells were transfected with control vector or LDHA overexpression vector for 48 hours. The expressions of LDHA were measured by Western blot. β-actin was a loading control. B. Glucose uptake (upper) and lactate product (lower) were measured in DLD-1 and LoVo cells with or without overexpressing LDHA. C. DLD-1 (upper) and LoVo (lower) cells were transfected with control vector or LDHA overexpression vector for 48 hours, cells were treated with rapamycin at 0, 1, 5, 10, 20, 40, 60 or 80 µM for 48 h, cell viability was determined by an MTT assay. *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$, compared with the control.

were upregulated at both protein and mRNA levels (**Figure 2D**, **2E**), indicating targeting upregulated glucose metabolism might sensitize rapamycin resistant cells.

Colon cancer cells with increased glycolysis show resistance to rapamycin

We next tested whether upregulating glycolysis by overexpression of glycolysis enzyme, LDHA could increase the resistance of colon cancer cells to rapamycin. Transfection of LDHA into DLD-1 and LoVo cells increased the LDHA expression levels and glucose metabolism (**Figure 3A**, **3B**). Therefore, we hypothesized colon cancer cells with elevated glycolysis were more resistant to rapamycin. We found in DLD-1 and LoVo cells, following rapamycin treatment at 0, 1, 5, 10, 20, 40, 60 or 80 µM for 48 h, the percentages of survival cells were significantly
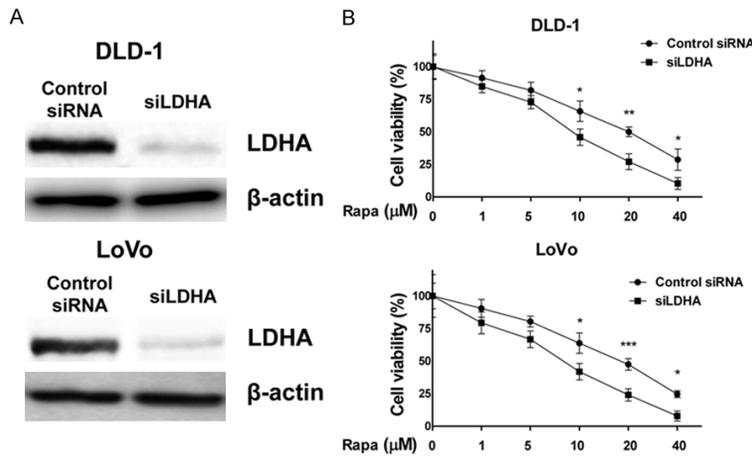
Figure 4. Knockdown LDHA by siRNA sensitizes colon cancer cells to rapamycin. A. DLD-1 (upper) and LoVo (lower) cells were transfected with control siRNA or siLDHA for 48 hours. The expressions of LDHA were measured by Western blot. β-actin was a loading control. B. DLD-1 (upper) and LoVo (lower) cells were transfected with control siRNA or siLDHA for 48 hours, cells were treated with rapamycin at 0, 1, 5, 10, 20 or 40 μM for 48 h, cell viability was determined by an MTT assay. *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$, compared with the control.

Figure 5. Synergistic inhibition of colon cancer cells by combination of rapamycin and glycolysis inhibitor. A. DLD-1 cells were treated with DMSO (control), rapamycin at 5, or 20 μM, Oxamate alone at 1 mM, rapamycin 5 μM plus Oxamate 1 mM or rapamycin 20 μM plus Oxamate 1 mM for 48 hours, cell viability was then determined by an MTT assay. B. LoVo cells were treated with DMSO (control), rapamycin at 5, or 15 μM, Oxamate alone at 1 mM, rapamycin 5 μM plus Oxamate 1 mM or rapamycin 15 μM plus Oxamate 1 mM for 48 hours, cell viability was then determined by an MTT assay. **, $P < 0.01$, ***, $P < 0.001$, compared with the control.

higher in LDHA overexpressing cells compared with control cells (**Figure 3C**). These results revealed that elevated glycolysis of colon cancer cells contributes to rapamycin resistance in a dose-dependent manner.

Inhibition of glycolysis by knockdown LDHA sensitizes colorectal cancer cells to rapamycin

The above results demonstrated upregulation of glycolysis rates renders colon cancer cells resistant to rapamycin. We therefore investigate whether inhibition of glycolysis could sensitize colon cancer cells to mTOR inhibitor. DLD-1 and LoVo cells were transfected siRNA to knockdown endogenous LDHA expressions (**Figure 4A**). DLD-1 and LoVo cells with or without knockdown LDHA were treated with rapamycin at 0, 1, 5, 10, 20 or 40 μM for 48 hours. Compared with control siRNA transfection, knocking down LDHA sensitizes DLD-1 and LoVo cells to rapamycin (**Figure 4B**). Viability rate of cells with knocking down LDHA was significantly lower than that of control siRNA transfected cells under rapamycin treatments (**Figure 4B**). Taken together, the above results demonstrated inhibition of glycolysis could re-sensitize rapamycin resistant colon cancer cells to rapamycin.

Combination of rapamycin and Oxamate shows synergistically inhibitory effects on colorectal cancer cells

We next investigated whether the combination of rapamycin and Oxamate could inhibit colon cancer cells synergistically. The two colon cancer cell lines (DLD-1 and LoVo) were treated with either Oxamate alone (1 mM), which is a glycolysis inhibitor through specific targeting LDHA [16], rapamycin alone (0, 5 or 20 μM) or in combination at a fixed ratio for 48 hours. We found Oxamate treatment alone at low concentrations (1 mM) did not significantly affect the cell survival of both cells (**Figure 5A**, **5B**). However, results illustrated a synergistic inhibition by the combined treatments of Oxamate and rapamycin on DLD-1 (**Figure 5A**) and LoVo (**Figure 5B**) colorectal cancer cells. In summary, our results demonstrated inhibition of glucose metabolism sensitizes colon cancer cells to rapamycin with a synergistic manner.

Discussion

mTOR has an essential role in the network sensing nutrition metabolism, inter- and intracellular growth signals, and cell growth and proliferation by promoting key anabolic processes [8-10]. Rapamycin is a natural, non-ATP competitive inhibitor of mTORC1 through binding to cytosolic FK binding protein (FKBP12) with high affinity [17]. Currently, several rapamycin analogs have been developed approved by the FDA for the treatment of cancer patients such as Everolimus (RAD001), which is an orally available derivative of Rapamycin, and Temsirolimus (CCI-779) [12]. Combined chemotherapy has been demonstrated to be an effect approach for improvement of chemo-resistant tumors and achieve survival benefits for cancer patients. Therefore, new combined chemotherapeutic drugs are broadly investigated for the treatment of patients with advanced colorectal cancer. In this study, we report a new combined treatment of colon cancer cells using rapamycin plus glycolysis inhibitor, Oxamate, demonstrating a synergistically inhibitory effect on colon cancer cells. However, the detailed molecular mechanisms remain unclear.

It has been reported that compared with normal cells, the metabolic properties of cancer cells diverge significantly [13]. The "Warburg effect" demonstrates that cancer cells are more dependent on anaerobic glycolysis to satisfy themselves with adequate energy and molecules [15]. Moreover, dysregulated cellular metabolism of cancer cells contributes to drug resistance [18]. Studies demonstrated that inhibition of cellular glycolysis could improve the efficacy of cancer therapy by targeting metabolic enzymes such as LDHA [19] and Hexokinase 2 [20]. Currently, the effects of mTOR inhibitor on cancer cells glucose metabolism are rare reported. A recent study illustrated that everolimus treatments inhibited glycolysis in Panc-1 human pancreatic cancer cells [21], suggesting a novel role of mTOR inhibitor in its antitumor effect as an inhibitor of glycolysis. Our results demonstrated rapamycin treatments significantly suppressed colon cancer cells glucose uptake and lactate product, consistent with the results from previous study [21]. In addition, we found rapamycin resistant colon cancer cells display increased glycolysis, providing evidences for the inhibitory

effects on cancer viability by the combination of glycolysis inhibitor and rapamycin. Thus, our work is the first study to determine the synergistic effectiveness by combining rapamycin and Oxamate in the treatments of colon cancer cells. This study highlights a potential therapeutic approach to target both mTOR pathway and glycolysis in colorectal cancer.

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Disclosure of conflict of interest

None.

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