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Augmented Pentose Phosphate Pathway Plays Critical Roles in Colorectal Carcinomas

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Key Words
Pentose phosphate pathway · Mammalian target of rapamycin · Aerobic glycolysis · Avemar · Reactive oxygen species · Colorectal carcinoma

Abstract
Glycolysis and the pentose phosphate pathway (PPP) are preferentially activated in cancer cells. Accumulating evidence indicated the significance of the altered glucose metabolism in cancer, but the implication for oncotherapy remains unclear. Here we report that the synthesis of glycolytic and PPP enzymes is almost ubiquitously augmented in colorectal carcinoma (CRC) specimens. The mammalian target of rapamycin (mTOR) inhibitor INK128 (300 nM) and phytochemical Avemar (1 mg/ml) inhibited the synthesis of PPP enzymes in CRC cell lines. INK128 (150–600 nM) and resveratrol (75–300 μM) inhibited aerobic glycolysis in the cell lines. INK128 (300 nM) and Avemar (1 mg/ml) decreased the NADPH/$NADP^+$ ratio as well as the GSH/GSSG ratio in the cell lines. Finally, per os administration of INK128 (0.8 mg/kg) or Avemar (1 g/kg) suppressed tumor growth and delayed tumor formation by transplantable CRC specimens derived from patients. Taken together, pharmacological inhibition of the mTOR-PPP axis is a promising therapeutic strategy against CRCs.

Background

Differentiated cells use two steps of glucose catabolism: glycolysis and oxidative phosphorylation (OXPHOS) [1]. Tumor cells and rapidly dividing cells, on the other hand, preferentially use glycolysis. Instead of transporting pyruvate into mitochondria for OXPHOS, such cells convert pyruvate into lactate for subsequent excretion (aerobic glycolysis or the Warburg effect [2, 3]). One significant feature of aerobic glycolysis is the activation of the pentose phosphate pathway (PPP), a reaction that bypasses mainstream glycolysis (online suppl. fig. 1; see www.karger.com/doi/10.1159/000369905 for all online suppl. material). In rapidly dividing cells, the PPP plays crucial roles in anabolic reactions, converting glucose into nucleotides or producing NADPH for fatty acid synthesis [4, 5]. NADPH, on the other hand, drives the recycling reaction of intracellular glutathione, one of the most important redox regulators [4, 5].

Recently, we have reported that the synthesis of succinate dehydrogenase subunit proteins A and B, the enzymes for OXPHOS, is frequently eliminated in hepatocellular carcinomas (HCCs) [6]. Importantly, the synthe-

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sis of the proteins for oncogenic pyruvate kinase type M2 and hypoxia-inducible factor-1α is not so prevalent in HCC specimens [6]. Instead, glucose-6-phosphate dehydrogenase (G6PD) and transketolase (TKT), the PPP enzymes, are transcriptionally induced by a redox regulator, i.e. the nuclear factor erythroid-2-related factor-2 (NRF2) [6].

The mammalian target of rapamycin (mTOR) signaling pathway integrates information about the cellular environment, such as nutrition, energy and redox conditions, thus controlling protein synthesis and cellular growth [7–9]. Through comprehensive analysis using knockout mouse embryonic fibroblasts, it has been revealed that mTOR complex 1 controls nearly all genes involved in mainstream glycolysis and the PPP [10]. The newly identified mTOR inhibitor, INK128, inhibits translation of genes including those encoding enzymes involved in glycolysis or the PPP [11].

In this study, we explored the feasibility of target therapy for colorectal carcinomas (CRCs), focusing on the mTOR-PPP axis. In CRC specimens, enzymes for both mainstream glycolysis and the PPP were frequently increased. INK128 and phytochemical Avemar inhibited the PPP reaction in CRC cell lines. In vivo, INK128 and Avemar suppressed tumor growth and delayed tumor formation by transplantable CRC specimens. Our findings confirm the relationship between mTOR and glucose metabolism in CRCs and highlight the mTOR-PPP axis as a novel therapeutic target.

Materials and Methods

Patients and Biospecimens

This study was approved by the Institutional Review Board of the Dokkyo Medical University Hospital (provided ID number: 26015), on the basis of the Ethical Guidelines for Clinical Research of the Ministry of Health, Labor and Welfare, Japan. Patients who were diagnosed as having CRC at the Dokkyo Medical University Hospital agreed to donate the surgically resected tumor specimens for research purposes. Each tumor specimen had accompanying nontumor mucosa and was used either for immediate primary culture or for protein extraction.

Cell Culture

Tissue specimens were washed vigorously with saline, minced with surgical scissors, and the minced tissue was further digested enzymatically in a collagenase/proteinase cocktail (0.1% collagenase L, Nitta Gelatin, Osaka, Japan, and 0.2% dispase, GIBCO, Carlsbad, Calif., USA, in HBSS, GIBCO) for 30 min in a reciprocating water bath shaker at 37°C. Undigested debris was removed by a 1,000- as well as a 500-μm nylon mesh, and finally cell clumps that did not pass through 100-μm nylon mesh were collected for primary culture [12]. The cell clumps formed cancer cell spheroids or otherwise became attached to the plastic dish on the following day with a success rate of almost 100% [12]. The culture medium was serum-free advanced DMEM/F-12 (GIBCO) supplemented with FGF-2 (10 ng/ml; ReproCell, Yokohama, Japan), ROCK inhibitor [13] (Y-27632, 10–20 μM; Wako Pure Chemical, Osaka, Japan), penicillin (100 mg/ml; Wako), streptomycin (0.1 mg/ml; Wako), gentamicin (50 μg/ml; Wako) and Fungizone (2.5 μg/ml; GIBCO). In order to prevent bacterial or fungal contamination, the medium was replaced with fresh medium 24 h after cell preparation. After 3 more days of culture, both attached and floating spheroids were collected, mixed with extracellular scaffold [14] and injected into nude mice subcutaneously. We did not adjust the cell number precisely at the first transplantation, since the ratio between attached and spheroid cells varied individually. DLD-1, LoVo, WiDr and COLO201 cell lines (for details, visit http://www.atcc.org and ATCC#CCL-221, CCL-229, CCL-218 and CCL-224, respectively) were purchased from JCRB Cell Bank (Osaka, Japan) and maintained under the conditions recommended by the supplier. The CACO-2 cell line (ATCC#HTB-37) was purchased from RIKEN BioResource Center (Ibaraki, Japan).

Protein Extraction and Immunoblotting

Cancer specimens were immersed in hypotonic solution (70 mM sucrose, 10 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM EGTA, 210 mM mannitol, 0.15 mM spermine, 0.75 mM spermidine, proteinase inhibitor cocktail; Wako and Roche Applied Science, Penzberg, Germany) and minced with surgical scissors. The tissue was then homogenized in a Dounce homogenizer and centrifuged. The supernatant contained cytosolic proteins, and the protein concentration of each specimen was measured by the Bradford protein assay (Bio-Rad, Hercules, Calif., USA). CRC cell lines were fixed with 10% trichloroacetic acid, and whole-cell proteins were dissolved with a solubilizer (7 M urea, 2 M thiourea, 2% Triton X-100; Wako). The procedures for immunoblotting have been described elsewhere [6]. GAPDH served as an internal control in HCCs [6] and in CRCs (less than two-fold alteration; fig. 1). Representative data are shown in the figures; all determinations were reproduced at least twice. Band intensity was quantified with IQTL software (version 8.1, GE Healthcare, Little Chalfont, UK), and differences of more than two-fold were considered 'increased/decreased'.

Drugs

Avemar is a fermented wheat germ extract that alters glucose metabolism in the T cell leukemia cell line [15]. It is currently used as an adjuvant therapy, and its efficacy has been proven in CRC [16] as well as melanoma [17]. Resveratrol is a phytochemical with chemopreventive activity, and its pharmacokinetics has been well studied in human CRC patients [18]. We purchased AveULTRA (99% pure Avemar; American BioSciences Inc., Blauvelt, N.Y., USA) and resveratrol (Wako) from the respective suppliers. The mTOR inhibitor INK128 was purchased from Sellek Chemicals (Houston, Tex., USA). CB83 (ChemBridge, San Diego, Calif., USA) is a newly identified G6PD inhibitor that displays superior specificity and affinity against G6PD [19]. However, the pharmacokinetics of CB83 is less well understood, and we did not observe any substantial growth-inhibitory effects using cell lines (data not shown). Per os administration of INK128 or Avemar has been well established [11, 20]. We followed the respective protocols. Briefly, INK128 is dissolved in 1-methyl-2-pyrrolidone (NMP; Sigma-Al-

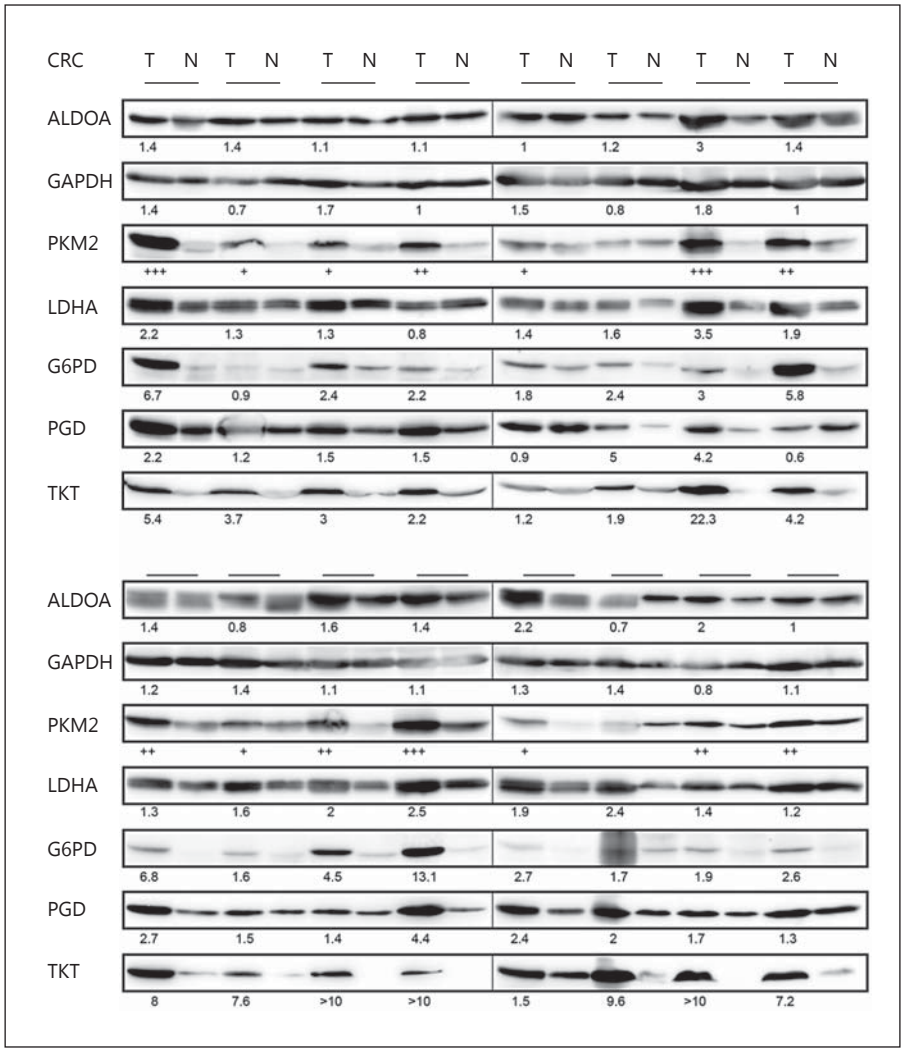

Fig. 1. Augmented protein synthesis of glycolytic enzymes in CRC specimens. Tissue samples from 16 different CRC patients are shown. Each well was loaded with 50 μg of cytoplasmic proteins, and Western blotting for glycolysis/PPP enzymes was performed. Tumor (T)/nontumor (N) paired samples were placed side by side. The band intensity was quantified by densitometry, and the tumor/nontumor ratios are presented below. Since synthesis of PKM2 in nontumor tissue is low, the level of PKM2 is presented as +, ++ or +++.

drich, St. Louis, Mo., USA) at a concentration of 2 mg/ml and diluted to 5% in polyvinylpyrrolidone K30 (PVP; Wako) to obtain final proportions of 5% NMP, 15% PVP, and 80% water. The dosages of INK128 and Avemar were 0.8 mg/kg/day and 1 g/kg/day, respectively.

Nude Mouse Tumor Serial Transplantation Assay

All experimental procedures were designed according to the guidelines of the animal facility at the Dokkyo Medical University and approved. Six-week-old female nude mice were purchased from Nippon Clea (Tokyo, Japan) and maintained under standard housing conditions. Among the injected primary culture specimens from 45 different patients, 11 formed tumors after 1–4 months. These subcutaneous tumors were again dissociated enzymatically and transplanted into other mice subcutaneously. Six cases were successfully transplanted more than three times (containing a sufficient number of cancer-initiating cells). Three cases were used for drug administration experiments (for data on each patient, see online suppl. table 1). When tumors were transplanted again, the amount of cells was adjusted by using the same tissue weight (500 mg). Divided tumors reproducibly formed tumors on the same day and grew at a similar speed, enabling comparison among test groups.

Primary Antibodies for Immunoblotting

For a list of primary antibodies used for immunoblotting in this study, see online suppl. table 2.

Measurement of Extracellular Glucose and Lactate

Glucose consumption and lactate excretion are affected by the total number of cells cultured. To exclude any secondary effect of growth arrest and cell death, we prepared cells in 24-well dishes at a confluent density and added each drug for 24 h. Using this simple procedure, we did not see any substantial cell death at any of the drug concentrations employed. Therefore, the changes in the amounts of glucose and lactate seemed to reflect alterations in the cellular metabolic status. We used a colorimetric assay kit to measure glucose (LabAssay Glucose, Wako) and L-lactate (L-Lactate Assay Kit, Cayman Chemical, Ann Arbor, Mich., USA) in conditioned culture medium.

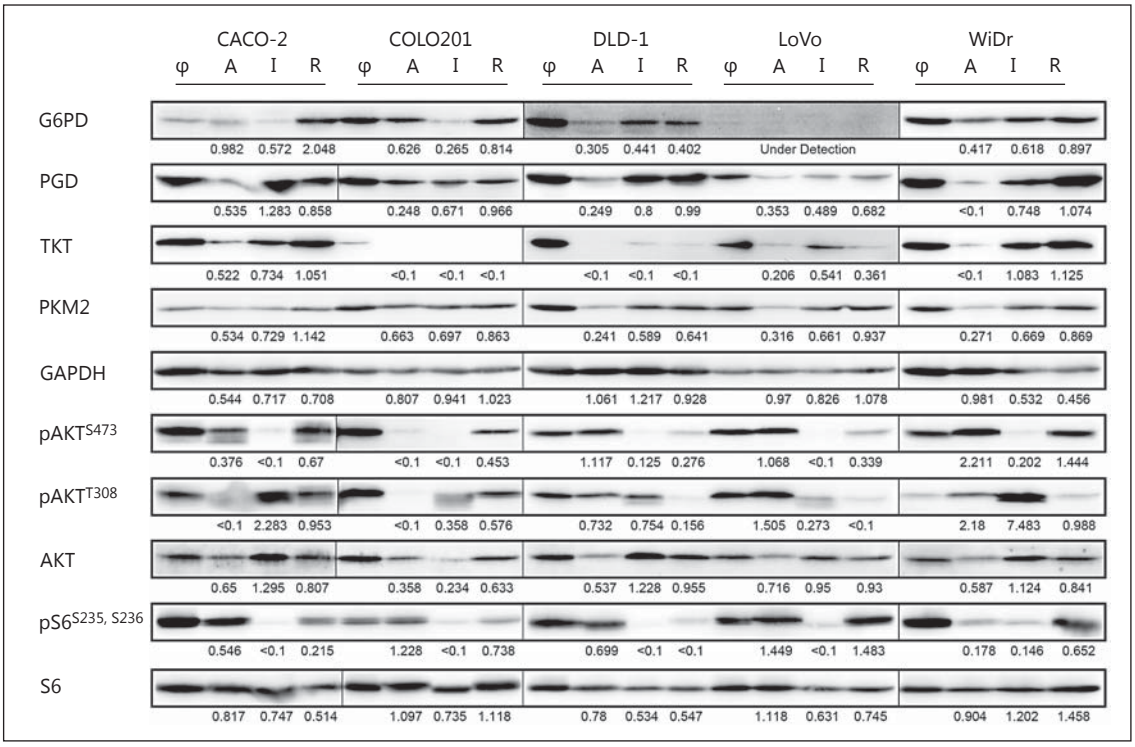

Fig. 2. INK128 and Avemar inhibit protein synthesis of PPP-related enzymes. Five CRC cell lines were used, and whole-cell lysates were extracted 24 h after each drug treatment [φ = control; A = Avemar 1 mg/ml; I = INK128 (mTOR inhibitor) 300 nM; R = resveratrol 150 μM]. Each well was loaded with 50 μg protein, and Western blotting was performed for glycolysis/PPP enzymes or phosphorylation of mTOR downstream proteins. The band intensity was quantified by densitometry, and the drug-treated/control ratios are presented below.

Measurement of Intracellular NADPH/NADP+ or GSH/GSSG Ratio

We purchased a colorimetric NADP+/NADPH quantification kit (BioVision, Milipitas, Calif., USA) and followed the manufacturer's instructions. For the measurement of the GSH/GSSG ratio, we either used the direct GSH detection method (GSH/GSSG Detection Assay Kit, Abcam, Cambridge, UK) for COLO201 or the cyclical regeneration assay with glutathione reductase (Microplate Assay for GSH/GSSG, Oxford Biomedical Research, Rochester Hiss, Mich., USA) for DLD-1. To determine whether drug treatment significantly affected the NADPH/NADP+ or GSH/GSSG ratio, one-way repeated-measures ANOVA was used. Subsequently, the difference resulting from each drug treatment was determined by paired Student's t test. Differences at $p < 0.05$ were considered statistically significant.

Results

Synthesis of Glycolytic Enzyme Proteins Is Augmented in CRC Specimens

Eleven out of 16 patients (69% of cases) displayed increased (more than two-fold relative to the nontumor counterpart) G6PD synthesis, and 13/16 patients (81% of cases) displayed increased TKT synthesis (fig. 1). Although a modest number of cases (6/16 patients) displayed increased phosphogluconate dehydrogenase (PGD) (fig. 1), this protein was readily detected in the nontumor mucosa. We observed almost comparable levels of aldolase A (ALDOA) synthesis in CRCs (fig. 1): 9/16 patients displayed PKM2 synthesis (++ or +++), and 5/16 patients displayed augmented synthesis of LDHA (fig. 1).

INK128 and Phytochemicals Inhibit mTOR Signaling and Synthesis of PPP Enzymes

INK128

First, we confirmed that the mTOR inhibitor INK128 inhibited phosphorylation of both mTORC1 and mTORC2 downstream target proteins (S6 and AKTS473, respectively) in all the cell lines tested (fig. 2). INK128 decreased the synthesis of PPP enzymes to various degrees (fig. 2): more than a two-fold decrease was observed for

G6PD in COLO201 (0.265 compared to untreated control) and DLD-1 (0.441), and more than a two-fold decrease was observed for PGD in LoVo (0.489). For TKT, more than a two-fold decrease was observed in COLO201 (<0.1) and DLD-1 (<0.1). INK128 did not affect the protein synthesis of either PKM2 or GAPDH (fig. 2).

Avemar

Next, we used the fermented wheat germ extract Avemar. It inhibited the phosphorylation of S6 in WiDr (0.178) and the phosphorylation of AKT^{S473} in CACO-2 (0.376) and COLO201 (<0.1) (fig. 2). Avemar markedly decreased the synthesis of PPP enzymes (fig. 2): more than a two-fold decrease was observed for G6PD in DLD-1 (0.305) and WiDr (0.417). For PGD, more than a two-fold decrease was observed in COLO201 (0.248), DLD-1 (0.249), LoVo (0.353) and WiDr (<0.1). For TKT, more than a two-fold decrease was observed in COLO201 (<0.1), DLD-1 (<0.1), LoVo (0.206) and WiDr (<0.1). Avemar decreased PKM2 in DLD-1 (0.241), LoVo (0.316) and WiDr (0.271), but none of the cell lines showed a change in GAPDH synthesis (fig. 2).

Resveratrol

Next, we used resveratrol for PPP inhibition. It inhibited the phosphorylation of S6 in CACO-2 (0.215) and DLD-1 (<0.1) and the phosphorylation of AKT^{S473} in COLO201 (0.453), DLD-1 (0.276) and LoVo (0.339). Resveratrol decreased the synthesis of PPP enzymes to various degrees (fig. 2): more than a two-fold decrease was observed for G6PD in DLD-1 (0.402), and for TKT, more than a two-fold decrease was observed in COLO201 (<0.1), DLD-1 (<0.1) and LoVo (0.361). Resveratrol hardly changed the synthesis of PGD, PKM2 or GAPDH protein, with a small number of exceptions (fig. 2).

INK128 and Resveratrol, but Not Avemar, Inhibit Aerobic Glycolysis in CRC Cell Lines

Most carcinoma cell lines consume glucose intensively and excrete lactate even in the presence of ambient oxygen in vitro (aerobic glycolysis). We examined whether INK128 or phytochemicals would suppress aerobic glycolysis. To see how much glucose is converted to lactate, we measured residual glucose in the conditioned culture medium and lactate excreted from the cells. INK128 markedly inhibited glucose consumption and decreased lactate excretion in a dose-dependent manner (fig. 3). This phenomenon was reproduced in all of the cell lines we used (representative data for COLO201, DLD-1 and LoVo are shown in fig. 3). Resveratrol displayed similar effects (fig. 3). In contrast, Avemar hardly affected glucose consumption or lactate excretion (fig. 3).

INK128 and Avemar Decrease the NADPH/$NADP^+$ and GSH/GSSG Ratios

G6PD drives the critical reaction for PPP and is one of the major enzymes that produce NADPH from $NADP^+$. Reduced NADPH drives the glutathione recycling reaction, converting oxidized GSSG dimer into two reduced GSH molecules (online suppl. fig. 2). Using two CRC cell lines, we measured intracellular NADPH and $NADP^+$ and calculated the ratio between them. The positive control G6PD inhibitor CB83 markedly lowered the NADPH/$NADP^+$ ratio in both COLO201 and DLD-1 (fig. 4). Similarly, Avemar decreased the NADPH/$NADP^+$ ratio in COLO201 and DLD-1 (fig. 4). INK128 produced a moderate decrease of NADPH/$NADP^+$ (statistically not significant) in COLO201 but not in DLD-1 (fig. 4). Resveratrol produced a moderate decrease of NADPH/$NADP^+$ (statistically not significant) in COLO201 but not in DLD-1 (fig. 4). Next, we measured intracellular GSH and GSSG and calculated the ratio between them. Avemar lowered the GSH/GSSG ratio in both COLO201 and DLD-1 (fig. 4). INK128 decreased the GSH/GSSG ratio in COLO201, but not in DLD-1 (fig. 4). Resveratrol decreased the GSH/GSSG ratio in COLO201 but not in DLD-1 (fig. 4). CB83 did not change the GSH/GSSG ratio.

INK128 and Avemar Suppress Tumor Growth and Delay Tumor Formation in Nude Mice

To observe the pharmacological effects in vivo, we employed a CRC specimen transplantation assay (Methods). To illustrate the individual differences in drug response, data for three representative cases are shown.

Case 1

Dissociated tumor cells were split and transplanted into 3 nude mice. Five days later, visible tumors appeared simultaneously and we randomly assigned each for comparison of drug effects. After oral administration of each drug for 16 days, the tumor weight in the control, Avemar and INK128 groups was 1.92, 1.33 and 0.97 g, respectively (fig. 5). Each tumor was further dissociated, part of which was transplanted into another mouse (2nd transplantation, online suppl. fig. 3), and the experiment was continued. After the 2nd transplantation and drug administration for 10 days, the tumor weight in the control and Avemar groups was 3.19 and 1.11 g, respectively (fig. 5). INK128 similarly decreased tumor weight 12 days

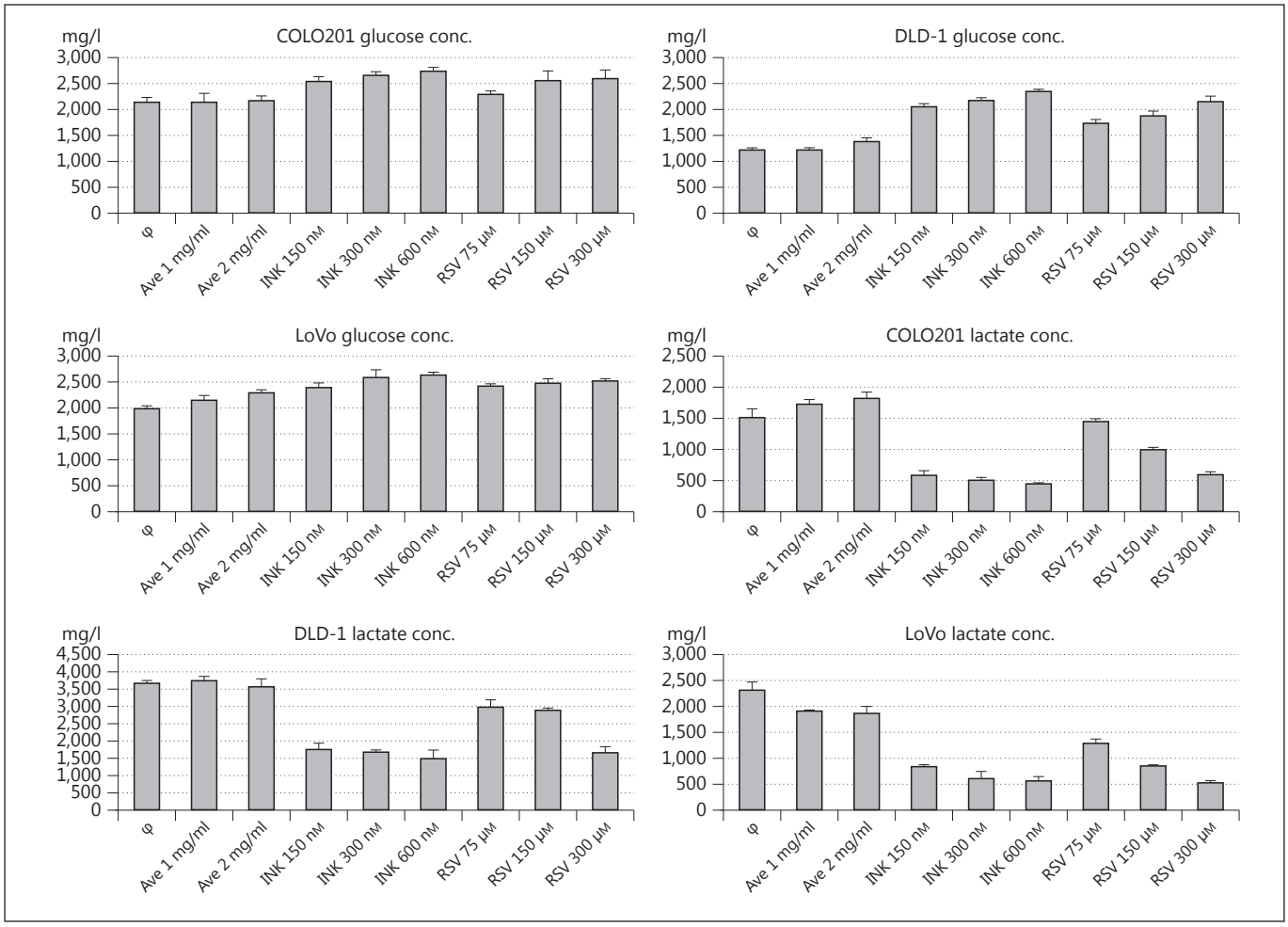

Fig. 3. INK128 and resveratrol inhibit glucose consumption and lactate excretion. Three CRC cell lines were used, and 24 h after treatment with each designated drug (φ = control; Ave = Avemar; INK = INK128; RSV = resveratrol), the concentrations of glucose (mg/l) and lactate (mg/l) in the conditioned culture medium were measured. Active glycolysis decreases residual glucose in the culture medium and increases excreted lactate. Therefore, the concentrations of glucose and lactate mostly show an inverse relationship. Mean values ± standard deviation (n = 3) are shown.

after drug administration (control:INK128 = 3.61:1.38 g; fig. 5). Each tumor was further dissociated, part of which was transplanted into another mouse (3rd transplantation). At the 1st and 2nd transplantations, we administered drugs after the appearance of visible tumors. At the 3rd transplantation, we started drug administration on the next day after tumor inoculation. The tumor in the INK128 group was split into two at the 3rd transplantation, but one of them did not form a tumor during 14 days of drug administration (fig. 5). Avemar did not show this phenomenon but repressed tumor growth similarly to the 1st and 2nd transplantations (control:Avemar = 2.67: 1.92 g; fig. 5). Finally, we purified proteins from each tumor (3rd transplantation) and measured the synthesis of proteins for PPP enzymes (fig. 6). Although INK128 inhibited the phosphorylation of mTOR downstream target proteins (fig. 6), neither Avemar nor INK128 changed the synthesis of mainstream glycolytic enzymes (fig. 6). INK128, however, slightly decreased the synthesis of G6PD and TKT protein (fig. 6).

Case 2

Similarly, tumor cells from another patient were split into three and transplanted into different mice. Three days thereafter, visible tumors appeared simultaneously, and we then started drug administration. After 16 days of

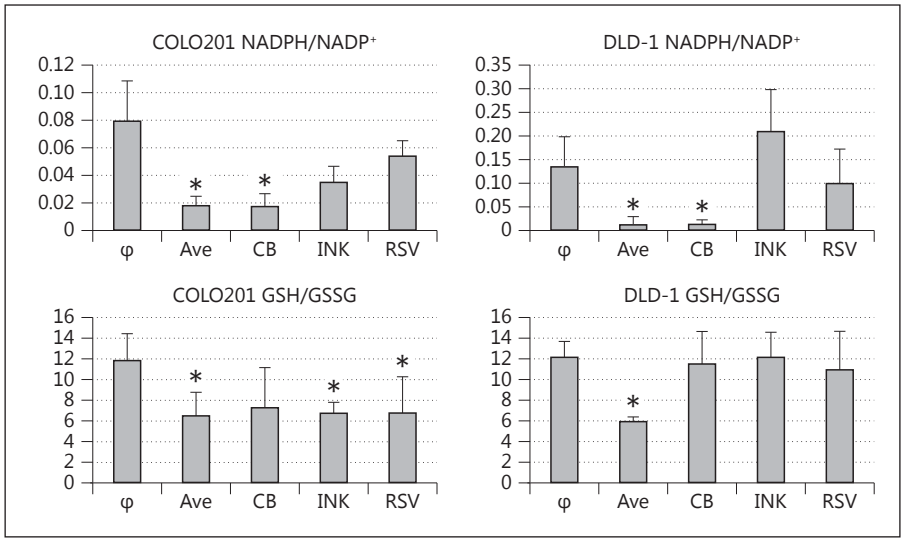

Fig. 4. INK128 and Avemar decrease the NADPH/NADP$^+$ and GSH/GSSG ratios. Two CRC cell lines were used, and 24 h after drug treatment [φ = control; Ave = Avemar 1 mg/ml; CB = CB83 (G6PD inhibitor) 12.5 μM; INK = INK128 300 nM; RSV = resveratrol 150 μM], whole-cell lysates were deproteinized and intracellular NADPH, NADP$^+$, GSH and GSSG were measured. Mean values ± standard deviation (n = 3 or 4) are shown. The asterisks indicate significant differences ($p < 0.05$, paired Student's t test) between the drug-treated and untreated control groups.

drug administration, Avemar and INK128 had decreased the tumor weight (control:Avemar:INK128 = 1.532: 0.574:0.764 g; fig. 5). At the 2nd transplantation, drug-treated tumors grew more slowly, and it took longer to obtain a substantial amount of tumor cells for the 3rd transplantation (control:Avemar:INK128 = 10:24:16 days from appearance to collection of each tumor; fig. 5). These respective tumors were further dissociated, parts of which were transplanted into other mice. Neither of the drugs delayed tumor formation but decreased the final tumor weight (control:Avemar:INK128 = 0.978:0.251: 0.281 g; fig. 5). After the 3rd transplantation, phosphorylation of AKT^{S473} was decreased by Avemar but not by INK128 (fig. 6). INK128 augmented the phosphorylation of AKT^{S473} and S6 protein (fig. 6). Avemar and INK128 decreased the synthesis of G6PD protein and Avemar decreased that of TKT protein (fig. 6). Neither Avemar nor INK128 changed the synthesis of mainstream glycolytic enzymes (fig. 6).

Case 3

Similarly, tumor cells from another patient were split into three and transplanted into different mice. Five days thereafter, visible tumors appeared simultaneously, and we then started drug administration. In this case, tumor cells were surprisingly resistant to drug treatment and INK128-treated tumors grew even larger at the 1st transplantation (control:Avemar:INK128 = 0.85:0.87:1.2 g; fig. 5). This was also the case for the 2nd transplantation (control:Avemar:INK128 = 0.787:0.796:0.73 g; fig. 5). However, when we continued the experiment until the 3rd transplantation, it took a longer time for drug-treated tumors to become visible in comparison with the control tumor (control:Avemar:INK128 = 5:12:13 days, respectively; fig. 5). As a result of this delay, the final tumor weight was much less at the same day of tumor collection (fig. 5). After the 3rd transplantation, Avemar augmented the phosphorylation of AKT^{S473} but decreased that of S6 (fig. 6). INK128 did not influence the phosphorylation of AKT^{S473} and S6 (fig. 6). Avemar and INK128 decreased the amount of G6PD, but none of the other enzymes were affected (fig. 6).

Discussion

A comprehensive review about the PPP [21] has indicated that the phytochemicals resveratrol and Avemar could inhibit the PPP. Considering the recently published report that mTOR integrates energy and redox conditions and mTORC1 controls genes for glucose metabolism in embryonic fibroblasts [10], it seemed appropriate to hypothesize that mTOR controls glycolysis and the PPP in CRCs as well. Indeed, we performed three in vitro assays using established CRC cell lines and found that the mTOR inhibitor INK128 and Avemar successfully inhibited PPP reaction in CRC cells (fig. 2, 4).

Usually, carcinoma cells in vitro consume large amounts of glucose, which is not fully oxidized into carbon dioxide even in the presence of ambient oxygen (aerobic glycolysis). The glycolytic final product, in turn, is discharged into the culture medium as lactic acid. As a

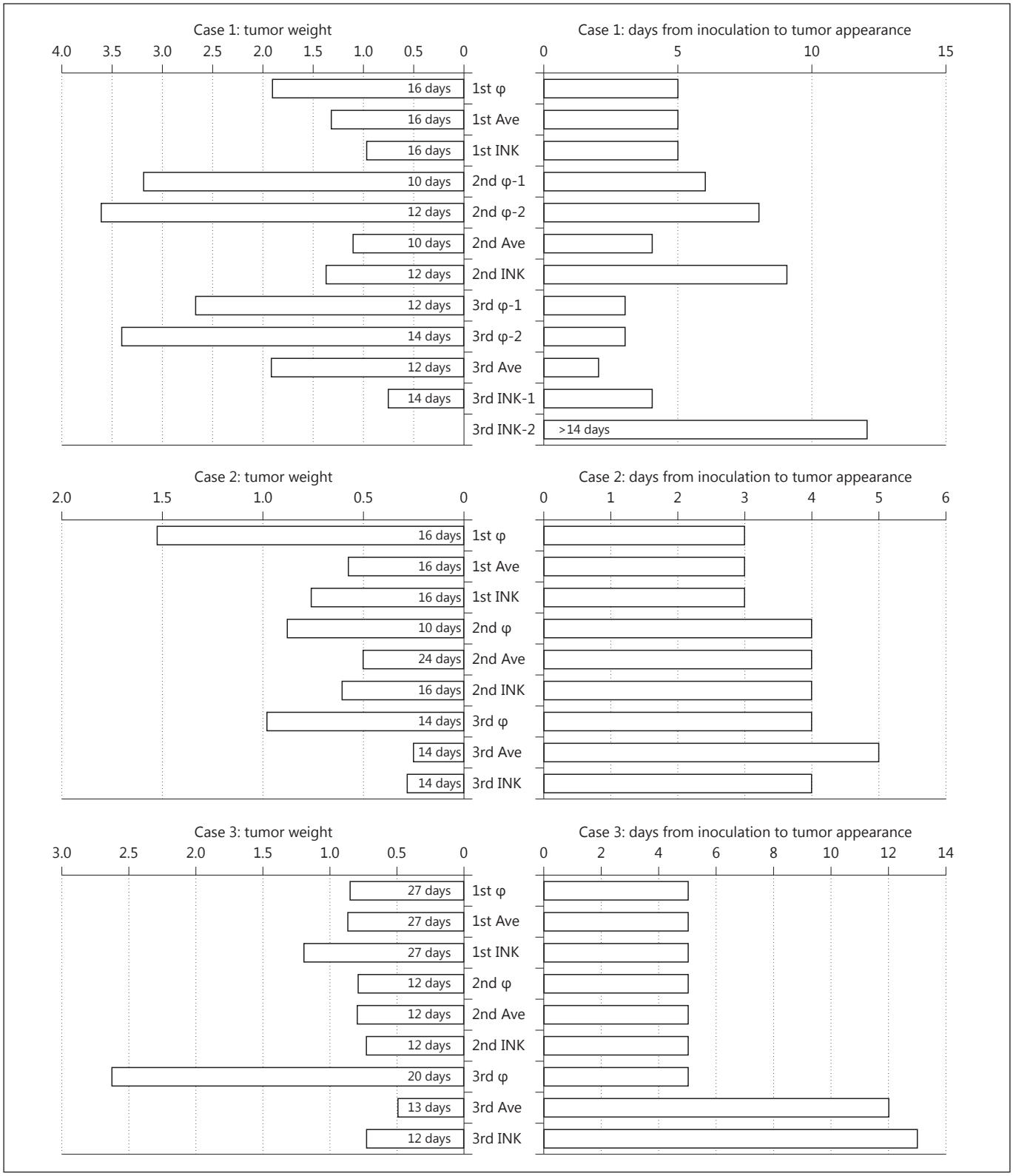

Fig. 5. INK128 and Avemar suppress tumor growth and delay tumor formation in nude mice. Left: Tumor weight; number of days written inside the bars indicates the period from the appearance of visible tumors until tumor collection. Right: Period from cell inoculation until appearance of visible tumors, indicating the latency.

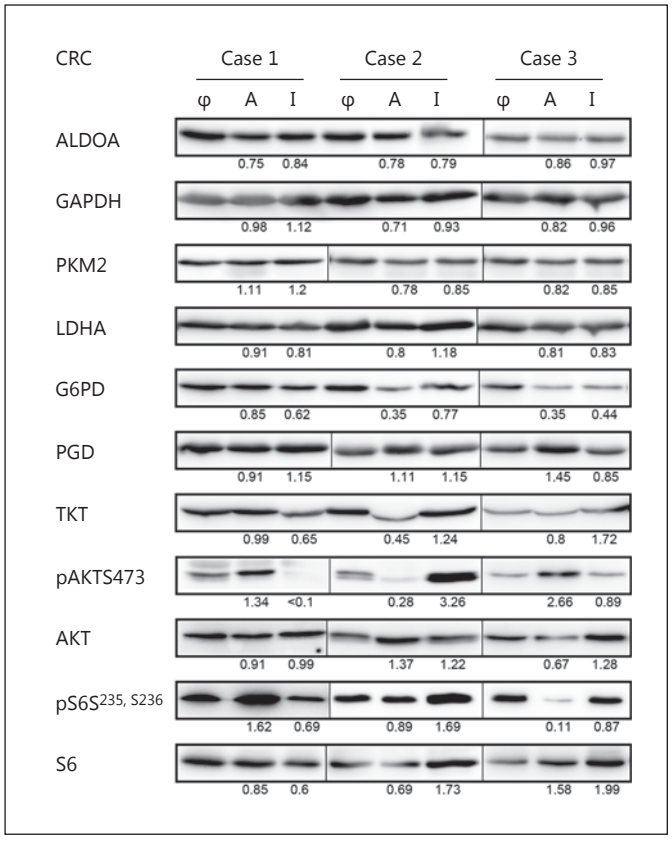

Fig. 6. INK128 and Avemar inhibited mTOR and protein synthesis of PPP enzymes. Tumors were collected after the 3rd transplantation and drug administration. Cytoplasmic proteins were extracted from each tumor (φ = control; A = Avemar 0.8 mg/kg; I = INK128 1 mg/kg). 50 μg proteins were loaded in each well. Western blotting for glycolysis/PPP enzymes or phosphorylation of mTOR downstream proteins was performed. The band intensity was quantified by densitometry, and the drug-treated/control ratios are presented below.

result, the pH indicator in the conditioned culture medium turns yellow (pH <6.8). When we started in vitro experiments, we noticed that the conditioned medium of INK128-treated cells remained red (i.e. pH approx. 7.5). Indeed, the amount of excreted lactic acid and remaining glucose in the conditioned medium remained unchanged after the treatment with INK128 (fig. 3). A similar phenomenon was also observed for resveratrol. However, Avemar did not have such a function (fig. 3) and neither did the G6PD inhibitor CB83 (data not shown). On the other hand, resveratrol poorly inhibited the synthesis of PPP enzymes (fig. 2), suggesting that aerobic glycolysis and the PPP do not necessarily cooperate with each other. Although the Warburg effect was originally described as aerobic glycolysis, we reported that activation of mainstream glycolysis is not prevalent in HCCs [6]. Although cross talk between two pathways does exist [4], it is possible that Avemar inhibits the PPP without affecting mainstream glycolysis and that resveratrol operates in the opposite way.

Glutathione is a peptide that buffers intracellular and extracellular redox potential. NADPH, a reduced form of $NADP^+$, is used to convert oxidized glutathione disulfide (GSSG) to reduced GSH [22]. The majority of intracellular NADPH is generated by the rate-limiting enzyme G6PD and downstream PGD in the PPP [23] (online suppl. fig. 1). Therefore, the PPP affects the NADPH/$NADP^+$ ratio, subsequently affecting the redox balance of glutathione indirectly (online suppl. fig. 2). Cancer cells utilize reactive oxygen species (ROS) to transmit signals which promote the epithelial-mesenchymal transition, cell motility, angiogenesis and inflammation [24]. On the other hand, chemotherapy or radiation therapy kills cancer cells by causing the production of ROS [25]. Therefore, it is first necessary for cancer cells to protect themselves from ROS induced by radiation or chemotherapy [26]. To achieve this, and to survive in the tumor microenvironment, it is believed that cancer cells develop counter-oxidant ability [27] (online suppl. fig. 2). For example, cancer cells produce a large amount of GSH and can neutralize ROS [25]. In certain tumors, activating mutations are found in the KEAP1-NRF2 pathway to counter oxidative stress [28–30]. We have also discovered that nuclear translocation of NRF2 induces the *G6PD* and *TKT* genes in HCCs [6]. The data in figure 4 suggest that PPP inhibition could potentially disable the antioxidant protection mechanism in CRCs.

Recently, we have developed an in vivo tumor transplantation model for patient-derived CRC specimens (online suppl. fig. 3). It is believed that the existence of cancer-initiating cells (also termed as cancer stem cells) is essential in order for tumors to replicate similar tumors in serial transplantation [31]. We used specimens that were transplantable for more than three passages in vivo, suggesting that those tumors contained a substantial number of cancer-initiating cells. Notably, INK128 and Avemar displayed growth-inhibitory effects against such malignant cases (cases 1 and 2; fig. 5). However, one specimen was completely resistant to both compounds (case 3; fig. 5). Another important finding was that INK128 and Avemar delayed tumor formation at the third course of transplantation and drug administration (cases 1 and 3; fig. 5). The rate-limiting step of metastasis is colonization of dispersed cancer cells [32]. Such cells must survive in

harsh microenvironments, and the transplanted tumor cells are considered to be under similar conditions. The mTOR-PPP axis could play an important role in successful colonization and metastasis. It should be mentioned, however, that an individual difference does exist even among the three different cases. Cases 1 and 2 are very fast-growing tumors, and Avemar or INK128 were effective in terms of growth control. On the other hand, INK128 and/or Avemar inhibited tumor appearance (colonization) of cases 1 and 3. Therefore, two different compensatory mechanisms exist: case 3 overcame growth inhibition and case 2 overcame colonization inhibition. Currently, we are designing new experiments to discriminate two aspects of mTOR-PPP inhibition.

Current target chemotherapies largely focus on specific oncogene pathways (e.g. WNT/β-catenin or RAS-MAPK in CRCs) [33]. Such an approach is limited by the high degree of intratumor heterogeneity and high somatic mutation rate, resulting in compensation of the genetic network [34, 35]. Glycolysis and the PPP are almost ubiquitously upregulated in CRCs (fig. 1), and targeting on the mTOR-PPP axis could be a strategy for disrupting 'nononcogene addiction' of CRCs. Classic chemotherapy or radiation induces ROS, and INK128 or Avemar would be expected to disable the protection system against ROS (fig. 4). Together with the facts that INK128 and Avemar are orally available drugs and currently at the clinical trial stage, designing some form of combination therapy with conventional anticancer drugs would be a feasible option.

In conclusion, the PPP is activated in CRC specimens and mTOR is one of the upstream signals. Pharmacological inhibition of PPP can be achieved by INK128 or Avemar both in vitro and in vivo. Therefore, targeting on the mTOR-PPP axis is a promising strategy for treatment of CRCs.

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Disclosure Statement

The authors declare that they have no competing interests.

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