Metformin enhances anti-tumor effect of L-type amino acid transporter 1 (LAT1) inhibitor

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A B S T R A C T

Background: In many cancer cells, L-type amino acid transporter 1 (LAT1) transports neutral amino acids with bulky side chain, which activate mammalian target of rapamycin (mTOR) to cause cell proliferation. An anti-diabetic drug, metformin, has been shown to activate AMP-activated protein kinase (AMPK), which leads to inhibition of mTOR. LAT1 inhibition in combination with metformin could result in more prominent suppression of mTOR activity.

Purpose: Anti-proliferative effect of a newly developed LAT1 specific inhibitor JPH203 in combination with metformin is evaluated in 2 head and neck cancer cell lines, Ca9-22 and HEp-2 cells and in nude mice inoculated with Ca9-22 cells.

Results and Discussion: By MTT assay, 0.5 mM metformin inhibited proliferation of Ca9-22 cells to 70% of control. In the presence of 100 μM JPH203, proliferation of Ca9-22 cells was inhibited to 60% of control. By combining these 2 drugs, proliferation of Ca9-22 was significantly inhibited to 40% of control. However, this regimen was not very effective against HEp-2 cells. This combination also suppressed in vivo growth of Ca9-22 cells in a xenotransplant model. A combination of anti-LAT1 drug with metformin may be an effective anti-proliferative therapy for certain subsets of cancers.

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1. Introduction

L-type amino acid transporter 1 (LAT1) is a twelve-transmembrane protein consisted of 512 amino acids (1). For LAT1 to be expressed at the cell membrane and functional it has to heterodimerize with another transmembrane protein 4F2hc via disulfide bond. LAT1 belongs to system L, which transports large neutral amino acids (1,2). Its expression in a healthy individual is either at the blood-organ barrier, i.e., brain, testis, and placenta, or in rapidly proliferating cells such as bone marrow cells and cells in embryonic tissues (1,3).

Notably, it is also expressed in various cancer cells, especially highly malignant ones (4). This cancer cell specific expression pattern can be used for diagnostic PET scan, where probes are substrates for LAT1 (5–8). LAT1 expression becomes higher as cancers become more malignant (9) and its expression levels have been shown to negatively correlate with length of survival in several cancers (10,11). Highly malignant cancers in general grow rapidly requiring energy (glucose) and biomolecular synthesis (amino acids and sugars for protein and nucleic acids) (12). One of the important signals leading to protein synthesis comes from activation of mammalian target of rapamycin (mTOR) pathway (12–14). Some amino acids (especially leucine) stimulate mTOR independent of growth factor signals (14). Between 2 different
pathway downstream of mTOR: mTOR complex 1 (mTORC1) and mTORC2, amino acids are shown to stimulate mTORC1 pathway (14). Because leucine is transported by system L and many cancer cells express LAT1 among system L transporters, LAT1 can be regarded as an upstream regulator of mTORC1 in such cancer cells and LAT1 inhibition has been reported to be promising a cancer therapy (15,16). Pan LAT inhibitor BCH is used in these studies (17). However, its lower potency and non-selectiveness prevents BCH itself from becoming a therapeutic agent. Recently, a more potent LAT1 specific inhibitor, JPH203, is shown to inhibit cancer cell growth in vitro in a human colon cancer cell line and in vivo in a xenotransplant model (18).

Independent of amino acid pathway, mTOR is also regulated by energy status of the cell (19). In energy depleted cells, AMP concentration increases while ATP concentration decreases. High concentrations of intracellular AMP activate AMP-activated protein kinase (AMPK), which leads to inhibition of mTOR activity (20). In a similar fashion as in energy depleted cells, an anti-diabetic drug metformin activates AMPK (19). Interestingly, there are several reports on anti-tumor effect of metformin (21,22) and an epidemiological study in type 2 diabetes patients shows that patients treated with metformin are less likely to develop cancer compared to patients without metformin (23).

Thus, addition of metformin to JPH203 would suppress mTOR pathway more strongly than either drug alone and this could lead to more effective inhibition on cancer cell proliferation.

2. Materials and methods

2.1. Reagents

An LAT1-specific inhibitor, JPH203 ((S)-2-amino-3-((4-((5-amino-2-phenylbenzo[d]oxazol-7-yl)methoxy)-3,5-dichlorophenyl)propanoic acid), was provided by J-Pharma Co., Ltd. (Yokohama, Japan) (18). Metformin (1, Dimethylbiguanide, Hydrochloride) was purchased from Merck KGaA (Darmstadt, Germany). All other reagents were purchased from Sigma unless otherwise indicated.

2.2. Cell culture

Two human head and neck squamous cell cancer lines, Ca9-22 and HEp-2, were used. Ca9-22 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cell lines were cultured in Eagle’s Minimum essential medium (MEM) with 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA). HEp-2, were used. Ca9-22 cells were purchased from the Japanese Collection of Research Bioresources (Osaka, Japan). HEp-2 cells were obtained from American Type Culture Collection (ATCC, Carlsbad, CA, USA). All the other reagents were purchased from Sigma unless otherwise indicated.

2.3. [14C]-leucine uptake assay

Cells were seeded on 24-well plates two days before experiment. After three times with standard uptake solution (125 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl2, 1.2 mM MgSO4, 25 mM HEPES, 1.2 mM KH2PO4 and 5.6 mM glucose, pH 7.4), they were pre-incubated with JPH203 and/or metformin for 10 min at 37 °C. For Na+ free condition, 125 mM choline chloride was used instead of 125 mM NaCl. Cells were incubated with 20 μM leucine including 1 μM [14C]-leucine for 2 min. After washing three times with ice-cold uptake solution, the cells were solubilized with 0.1 N NaOH, added OptiPhase Supermix (PerkinElmer, Waltham, MA, USA) and the radioactivity was measured by a liquid scintillation counter.

2.4. Measurement of protein concentration

Protein concentration was measured using Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as a standard.

2.5. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total cellular RNA was isolated and purified from cells using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instruction. RNA samples were treated with RNase-free DNase RQ1 (Promega Corporation, Madison, WI, USA) and purified by suspending into phenol/chloroform/isooamyl alcohol (25:24:1) solution. The first-strand complementary DNA (cDNA) was synthesized using MuLV Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA) with oligo dT primer. Real-time PCR for LAT1, LAT2, LAT3, LAT4 and 4F2hc was conducted with 7300 Real-Time PCR system (Life Technologies, Carlsbad, CA, USA) Designed primers and TaqMan probes were shown in Table 1. GAPDH was quantified with TaqMan GAPDH control Reagents (Human) (Life Technologies, Carlsbad, CA, USA). All samples were analyzed in triplicates.

2.6. Immunocytochemistry

Cells were washed with PBS containing 1 mM MgCl2 and 0.1 mM CaCl2 (PBS−), fixed in cold methanol for 7 min and washed 3 times with PBS++. Fixed cells were incubated with permeabilization buffer (0.1% BSA, 1% Triton X-100 in PBS−) for 15 min and blocked in goat serum dilution buffer (GSD), (10% goat serum, 1% Triton X-100, 10 mM glycine in PBS++) for 60 min. Cells were incubated with primary antibodies diluted in GSD buffer overnight at 4 °C and washed 3 times with permeabilization buffer, then incubated with anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 568 conjugated IgG (Life Technologies, Carlsbad, CA, USA) diluted in GSD buffer for 1 h, after which they were washed 3 times in PBS++ and once in water. Cells were mounted in fluorescence mounting medium (DAKO JAPAN, Tokyo, Japan) and was visualized with an Olympus Fluoview FV500 laser confocal microscope (Olympus, Tokyo, Japan).

2.7. Cell viability assay

Ca9-22 and HEp-2 cells were seeded in 24-well plate in normal growth medium. After 2 days of incubation, cells were treated with JPH203 and/or metformin, followed by incubation for 7 days. Cell

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<tr>
<th>Transporters</th>
<th>Primers and probes used for Taqman PCR</th>
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<tr>
<td>LAT1 forward</td>
<td>GGAAGGGTGTGCTGCTAACTCT</td>
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<tr>
<td>reverse probe</td>
<td>TCCAGATTGCATTCACTTCCATAG</td>
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<tr>
<td>LAT2 forward</td>
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</tr>
<tr>
<td>reverse probe</td>
<td>GTGATCATACGACGACGACATCEC</td>
</tr>
<tr>
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<td>CCCCAATCAGGCGACTTGT</td>
</tr>
<tr>
<td>reverse probe</td>
<td>GTACGTTGGTCGTCGATGGTTT</td>
</tr>
<tr>
<td>LAT4 forward</td>
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<td>reverse probe</td>
<td>CGTACGCAATCGACCAGAGA</td>
</tr>
<tr>
<td>4F2hc forward</td>
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</tr>
<tr>
<td>reverse probe</td>
<td>R6GGTGGACATGCAGTTGAGG</td>
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Table 1

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viability was measured by MTT assay. 0.45 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Life Technologies, Carlsbad, CA, USA) was added to the cells. The cells were incubated for 4 h. After aspiration of medium containing MTT, isopropanol with 0.04 N HCl was added and absorbance was measured at 570 nm using SH-9000Lab (CORONA ELECTRIC, Ibaraki, Japan).

2.8. Western blot analysis

Ca9-22 and HEp-2 cells were seeded in 6-well plates in the normal growth medium and allowed 2 days for growth. In the case of phospho-p70S6 kinase (p70S6K) detection, serum was depleted overnight. Then Amino acids were depleted 2 h by incubating the cells in Hank's Balanced Salt Solution (Life Technologies, Carlsbad, CA, USA). Medium were then changed to either, growth medium without serum but with amino acids (positive control; 56k should be phosphorylated), the same amino acid replete medium containing 0.5 mM metformin and/or 100 μM JPH203 and the cells were incubated another 30 min. In the case of phospho-acetyl-CoA carboxylase (pACC) detection, the cells were incubated overnight in serum free medium in the absence (control) or presence of 0.5 mM metformin. After washing with ice-cold PBS, the cells were harvested by scraping in lysis buffer (150 mM NaCl, 0.5 mM EDTA, 1% Triton X-100 and 50 mM Tris–HCl, pH 7.4) with cOmplete, Mini, EDTA-free protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). After incubation at 4 °C for 1 h, cell lysates were centrifuged for 30 min at 14,000 rpm. The supernatants (30 μg of protein) were subjected to SDS–PAGE, followed by transferring to Immobilon-P PVDF membrane (Merck KGaA, Darmstadt, Germany). The membrane was incubated with either primary antibodies: phosphor ACC, phosphor 56K from Cell Signaling Technology (Danvers, MA, USA), β-actin (Clone AC-15), LAT1 (TRANS GENIC, Kumamoto, Japan) and 4F2hc (Santa Cruz Biotechnology, Dallas, Texas, USA) antibody. After washing three times with Tris buffered saline with Tween 20 (TBS-T) (137 mM sodium chloride, 20 mM Tris, 0.1% Tween-20), these membranes were incubated with goat anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The membrane was washed three times with TBS-T. Chemiluminescence detection images and densitometry were obtained with ImageQuant LAS-4000 (Fujifilm, Tokyo, Japan).

2.9. Apoptosis assay

Ca9-22 and HEp-2 cells were seeded in 96-well plates in the normal growth medium and allowed 2 days for growth. Cells were treated with JPH203 and/or metformin, followed by incubation for 2 days. Apoptosis was measured by the Caspase-Glo® 3/7 Assay kit from Promega Corporation (Madison, WI, USA) according to its protocol.

2.10. Xenograft model anti-tumor assay

Nude mice, BALB/cSlc-nu/nu (Japan SLC, Inc, Hamamatsu, Japan), was used for xenotransplantation. Cyclophosphamide was administrated by intraperitoneal injection at 100 mg/kg one day before transplantation of Ca9-22 cells. Mixture of 0.5 × 10^5 Ca9-22 cells and Matrigel™ basement membrane matrix (BD Biosciences, Bedford, MA, USA) was injected into nude mice subcutaneously. Eight mice were prepared for each condition. After 4 weeks, intravenous administration of JPH203 (6 mg/kg) and/or oral administration of metformin (300 mg/kg) was started. These drugs were administered once a day and continued for 14 days. Tumor volume (volume = (width)^2 × length/2) was measured with micrometer calipers and observed over the treatment time. Toxic effect was evaluated by necropsy after administration of drugs.

2.11. Animal ethics statements

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Ministry of Education, Culture, Sports, Science and Technology-JAPAN. The protocol was approved by Institute of Laboratory Animals, Graduate School of Medicine, Kyorin University (The Experimental Animal Ethics Committee in Kyorin University, permit number 92).

2.12. Statistical analysis

Welch’s t-test was used to compare all experimental results. A P-value less than 0.05 was considered significant.

3. Results

3.1. LAT1 was mainly responsible for leucine uptake in head and neck cancer cell lines, Ca9-22 and HEp-2

Because leucine is a neutral amino acid with bulky side chain and known to stimulate mTOR most effectively (24), leucine was used as a prototypical substrate. It has been shown that leucine is transported by Na^+–dependent or Na^+–independent systems (25). As shown in the Fig. 1A, presence of Na^+ did not change leucine uptake to a major degree in both cells; Na^+–independent leucine uptake was 90% of total uptake in Ca9-22 cells and 81% in HEp-2 cells, indicating that Na^+–independent system L is mainly responsible for leucine uptake in these cells. Among 4 members of system L transporters, only LAT1 was expressed in a significant amount in both Ca9-22 and HEp-2 cells by qRT-PCR (Fig. 1B). LAT1 and co-expressing molecule 4F2hc proteins were detected in these cells by western blotting (Fig. 1C). It should be noted that both mRNA and protein level expression of LAT1 was much higher in Ca9-22 cells than HEp-2 cells; at mRNA level, LAT1 expression of Ca9-22 cells was 6-fold higher than that of HEp-2 cells (Fig. 1B), and densitometry of LAT1 signals in a western blot showed that LAT1 protein in Ca9-22 cells were 12-fold higher than that in HEp-2 cells (Fig. 1C). Immunocytochemical analysis confirmed that cell surface expression of these proteins in Ca9-22 and HEp-2 cells (Fig. 1D).

3.2. LAT1 inhibition led to suppression of both leucine uptake and proliferation

Consistent with the aforementioned result that LAT1 was the major Na^+–independent system L transporter expressed in these cell lines, JPH203, a specific inhibitor for LAT1, blocked up to 78% (10 μM) or 85% (100 μM) of leucine uptake into Ca9-22, 62% (10 μM) or 71% (100 μM) of leucine uptake into HEp-2 cells, respectively (Fig. 2A).

As shown in Fig. 2B, addition of JPH203 to the culture suppressed proliferation of Ca9-22 or HEp-2 cells with similar potency in a dose-dependent fashion with IC_50 (half maximal inhibitory concentration) of low 100 μM range.

Expression of LAT1 and 4F2hc after treatment with JPH203 for 30 min and 7 days was examined (Fig. 2C). LAT1 or 4F2hc expression in Ca9-22 and HEp-2 cells was not changed at 30 min and slightly upregulated at 7 day.
3.3. Metformin inhibited Ca9-22 and HEp-2 cell proliferation

Metformin is an anti-diabetic drug and activates AMPK by increasing cytosolic AMP concentrations (26). Activation of AMPK could suppress cell proliferation through inhibition of mTOR.

Decreased viabilities of Ca9-22 or HEp-2 cells were observed in the presence of metformin (Fig. 3A). When Ca9-22 or HEp-2 cells were cultured in the presence of various concentrations of metformin, their growth measured by MTT assay was suppressed to the similar extent, IC50 being around 1 mM.

Western blot was performed to confirm AMPK activation by metformin. Acetyl-CoA carboxylase (ACC) is one of the well-documented substrates of AMPK. As shown in Fig. 3B, metformin increased phosphorylation of ACC in both Ca9-22 and HEp-2 cells.

3.4. Effect of metformin-JPH203 combination on cell proliferation was cell-type dependent

In order to elucidate the synergism between metformin and JPH203, the lowest concentration of each drug that consistently suppressed growth of both Ca9-22 and HEp-2 cells was chosen. In Ca9-22 cells, 0.5 mM of metformin suppressed their growth to ~70% of control and 10 μM of JPH203 suppressed their growth to ~80% of control (Fig. 4A). When the cells were cultured in the presence of both metformin and JPH203, their growth was suppressed to ~40% of control (Fig. 4A), which was expected if these 2 drugs act independently (this concentration of metformin suppressed proliferation to ~70% of control and this concentration of JPH203 to ~80% of control, thus 60/80 = ~40% of control would be expected if these 2 drugs act independently). In HEp-2 cells, the same concentration of metformin and JPH203 suppressed their growth to ~80% and ~75% of control, respectively (Fig. 4B). Co-administration of metformin and JPH203 suppressed their growth to ~70% of control, which was statistically significant compared to the growth observed in the presence of JPH203 alone (Fig. 4B). However, unlike Ca9-22 cells, the degree of growth suppression by combining 2 drugs was less than expected (~60% of control was expected if these 2 drugs act independently).

Since intracellular depletion of leucine was expected to suppress mTOR signaling via mTORC1, phosphorylation status of mtOR itself and its downstream effector p70S6K were examined in the absence/presence of JPH203 in Ca9-22 and HEp-2 cells (Fig. 5). Removal of amino acids from culture medium led to dephosphorylation of mTOR and p70S6K. Phospho-p70S6K level was greatly decreased in the presence of JPH203 in Ca9-22 cells, while such suppression was not detectable in HEp-2 cells.

It has been reported that inhibition of LAT1 by BCH and JPH203 leads apoptosis on cancer cell lines (17,27). We tested the effect of metformin and JPH203 on caspase activity to check whether apoptosis is induced after drug treatment (Table 2). Metformin...
alone had a little effect on caspase activity in both HEp-2 and Ca9-22 cells. JPH203 treatment led to activation of caspase. Combination of JPH203 and metformin decreased the level of caspase activation compared to JPH203 alone.

3.5. Metformin-JPH203 combination suppressed Ca9-22 cell growth in vivo in a xenotransplant model

Combination effect of metformin and JPH203 was investigated in vivo using Ca9-22 cells transplanted into nude mice (Fig. 6). At the end of 14-days treatment 6 mg/kg JPH203 had minimal, if any, effect on tumor growth and 300 mg/kg metformin tended to inhibit tumor growth, although this suppression did not reach statistical significance compared with control treatment. Combination of metformin and JPH203 inhibited tumor growth more than each drug alone and to a degree statistically significant compared with control treatment. Necropsy of these animals did not reveal any toxic effects.

4. Discussion

Head and neck cancer occurs mainly in elderly people. In such patients, a strong cytotoxic chemotherapy or radical surgery decreases quality of life and it is often impossible to take these therapies even when they are deemed to be curative. Less toxic anti-cancer treatment regimens would be more desirable. This study demonstrated that the main route of leucine uptake in 2 head and neck cancer cell lines (Ca9-22 and HEp-2 cells) was via system L and LAT1 was the only system L transporter expressed in a significant quantity in these cells. A newly developed LAT1 specific inhibitor JPH203 not only inhibited leucine uptake and proliferation of these cells, but also induced apoptosis. But it is not as potent as classic cytotoxic chemotherapy drugs. Metformin activated AMPK and exhibited growth inhibitory effect of Ca9-22 and HEp-2 cells with similar potency although a much higher concentration was required than diabetes treatment. If metformin and JPH203 would work synergistically, it might be possible to enhance potency of JPH203 and avoid toxicity of metformin. When JPH203 and metformin were combined, growth suppression was enhanced in both cells but the degree of inhibition was higher in Ca9-22 cells. In Ca9-22 cell xenotransplant models, similar results were obtained and no toxicity was observed after administration of two drugs. It is promising that the combination of sub IC50 concentrations of metformin and JPH203 led to more than 50% growth suppression in Ca9-22 cells (Fig. 4). The fact that metformin and JPH203 acted in an additive fashion rather than synergistic in Ca9-22 cells (Fig. 4A)
suggests that metformin and JPH203 inhibited growth via independent mechanism. Metformin was shown to activate AMPK (Fig. 3B) and AMPK is known to suppress mTORC1 activity via activation of TSC2 (28). JPH203 inhibited leucine uptake (Fig. 2A), cell proliferation (Fig. 2B), and mTOR activity (mTORC1 activity) (Fig. 5) in Ca9-22 cells. A recent study revealed that amino acids are sensed by Rag GTPases at the lysosomal membrane and mTORC1 is recruited to the surface of the lysosome (29). Thus, it is likely that input from Rag and TSC2 is processed independently in mTORC1.

Why was the combination of JPH203 and metformin in HEp-2 cells not as effective (Fig. 4) despite the similar potency of these drugs as in Ca9-22 cells (Figs. 2B and 3A)? Effect of metformin is similar in HEp-2 and Ca9-22 cells (Fig. 3B). In contrast, regulation of LAT1-amino acid-mTORC1-p70S6K pathway appears different between these cells; suppression of p70S6K phosphorylation by JPH203 was less in HEp-2 cells than in Ca9-22 cells (Fig. 5). This...
observation led us to speculate that relative contribution of mTORC1 pathway in proliferation may be less in HEP-2 cells than Ca9-22 cells. However, JPH203 was as effective in HEP-2 cells as in Ca9-22 cells (Fig. 2B), suggesting that mechanisms other than inhibition of mTORC1-p70S6K might be acting for JPH203 in HEP-2 cells. Such mechanism requires further investigation. Regardless of the mechanisms, it should be emphasized that not all cancers are likely to respond to this combination therapy. Therefore, it is very important to find biomarkers predicting responsiveness to the combination therapy and we are in the process of investigation.

As shown in Table 2, metformin by itself had no effect on apoptosis in HEP-2 cells while it had slight suppressive effect on that in Ca9-22 cells. JPH203 induced apoptosis and combination treatment decreased caspase activation compared with that in JPH203 treatment in both of these cells, suggesting that metformin has anti-apoptotic effect in this context. These results are not consistent with other literature that show pro-apoptotic effect of metformin (30–32). However, metformin was also reported to show anti-apoptotic effect (33) in certain context. This may be one of the reasons that metformin and JPH203 did not act synergistically in these cells.

It is encouraging that the combination of JPH203 and metformin inhibited in vivo cancer cell growth in a xenotransplant model (Fig. 6). The absolute degree of growth suppression (~80% of control) might not be as dramatic as classic cytotoxic anti-cancer drugs. However, the treatment appeared free from toxic side effects. Mild tumor growth suppression plus no significant toxic side effects might translate into superior quality of life in cancer patients.

5. Conclusions

Metformin additively enhanced anti-proliferative effect of an LAT1 specific inhibitor, JPH203, in Ca9-22 head and neck cancer cell line both in vitro and in vivo. In this cell line, AMPK activation by metformin and amino acid depletion by JPH203 may independently act on mTORC1 and suppress its activity.

Conflict of interest statement

H Endou is employed by J-Pharma Co. Ltd. Other authors have no COI to disclose.

Acknowledgments

We thank members of the lab group for technical assistance, comments and helpful discussions.

References


Table 2

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<th>Ca9-22 (%)</th>
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<tr>
<td>Captisol</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Metformin</td>
<td>106.5 ± 2.7</td>
<td>84.4 ± 3.2</td>
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<tr>
<td>JPH203</td>
<td>204.9 ± 3.3</td>
<td>190.5 ± 12.1</td>
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<td>Metformin + JPH203</td>
<td>179.4 ± 5.2</td>
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Fig. 6. Effect of JPH203 and metformin on xenograft tumor progression. Ca9-22 cells were injected into nude mice subcutaneously and JPH203 and metformin was given by intravenous injection and oral administration, respectively, once a day and continued for 14 days. The drug treatment was started after 4 weeks of tumor inoculation. Tumor volume was calculated as volume – (width)² × length/2 and the data were shown in ratio compared to the volume just before the drug administration, which was designated as 100%.

*P < 0.05.


