Efficacy of Dual Inhibition of Glycolysis and Glutaminolysis for Therapy of Renal Lesions in Tsc2 +/- Mice

Abstract

Tuberous sclerosis is caused by mutations in the TSC1 or TSC2 gene and characterized by development of tumors in multiple organs including the kidneys. TSC-associated tumors exhibit somatic loss of the second allele of the TSC genes, leading to aberrant activation of the mechanistic target of rapamycin (mTOR) signaling pathway. Activation of mTOR complex 1 (mTORC1) causes addiction to glucose and glutamine in Tsc1 +/- or Tsc2 +/- mouse embryonic fibroblasts (MEFs). Blocking of glutamine anaplerosis in combination with glycolytic inhibition causes significant cell death in Tsc2 +/- but not Tsc2 +/- MEFs. In this study, we tested efficacy of dual inhibition of glycolysis with 3-BrPA and glutaminolysis with CB-839 for renal tumors in Tsc2 +/- mice. Following 2 months of treatment of Tsc2 +/- mice from the age of 12 months, combination of 3-BrPA and CB-839 significantly reduced overall size and cellular areas of all renal lesions (cystic/papillary adenomas and solid carcinomas), but neither alone did. Combination of 3-BrPA and CB-839 inhibited mTORC1 and the proliferation of tumor cells but did not increase apoptosis. However, combination of 3-BrPA and CB-839 was not as efficacious as rapamycin alone or rapamycin in combination with either 3-BrPA or CB-839 for renal lesions of Tsc2 +/- mice. Consistently, rapamycin alone or rapamycin in combination with either 3-BrPA or CB-839 had stronger inhibitory effects on mTORC1 and proliferation of tumor cells than combination of 3-BrPA and CB-839. We conclude that combination of 3-BrPA and CB-839 may not offer a better therapeutic strategy than rapamycin for TSC-associated tumors.

Introduction

Tuberous sclerosis is caused by mutations in the TSC1 or TSC2 gene and characterized by development of tumors in different organs [1]. Over 80% of TSC patients develop multiple and bilateral angiomyolipomas in the kidneys that are the leading cause of adult deaths from the disease. Around 50% of TSC patients have renal cysts, and approximately 4% of TSC patients suffer from renal carcinomas. The TSC1/TSC2 complex downregulates the mechanistic target of rapamycin (mTOR) signaling pathway through its GTPase activating protein (GAP) activity towards the small G-protein Rheb (Ras homologue enriched in brain) [2]. TSC-associated tumors exhibit somatic loss of the second allele of the TSC genes, giving rise to aberrant activation of the mTOR signaling pathway in human and mouse [3,4]. Rapamycin and its analogs (rapalogs) are allosteric mTOR inhibitors and reduce size of TSC-associated tumors, but these tumors regrow upon cessation of treatment [5–7]. Activation of mTOR complex 1 (mTORC1) promotes glycolysis and glutaminolysis in Tsc2 +/- mouse embryonic fibroblasts (MEFs) that are addicted to glucose and glutamine [8,9]. Glycolytic inhibition suppresses tumor growth in a mouse model transplanted with Tsc2 +/- rat tumor cells [10]. Blocking of glutamine anaplerosis in combination with glycolytic inhibition causes significant cell death in Tsc2 +/- but not Tsc2 +/- MEF cells [9]. Dual inhibition of glycolysis and glutaminolysis may provide a new approach to treating tumors lacking TSC1 or TSC2.

The pyruvate analog 3-bromopyruvate (3-BrPA) is an efficient inhibitor of glycolysis through alkylating glycolytic enzymes,
particularly hexokinase II (HK2) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [11]. Being well tolerated, 3-BrPA is very effective for treating liver carcinomas in rabbit and mouse models [12]. CB-839 is a potent blocker of glutaminolysis by selectively inhibiting glutaminase (GLS). Preclinical studies have suggested effective antitumor activity of CB-839 in various types of cancer [13–16].

In this study, we tested efficacy of dual inhibition of glycolysis with 3-BrPA and glutaminolysis with CB-839 for renal tumors in Tsc2+/- mice. We found that combination of 3-BrPA and CB-839 significantly reduced overall size and cellular areas of all renal lesions including cystic/papillary adenomas and solid carcinomas, but neither alone did. However, combination of 3-BrPA and CB-839 was not as efficacious as rapamycin alone or rapamycin in combination with either 3-BrPA or CB-839 for renal tumors of Tsc2+/- mice.

Materials and Methods

Animal Procedures

Animal procedures were performed in accordance with the UK Home Office guidelines and approved by the Ethical Review Group of Cardiff University. Tsc2+/- mice were described previously [17]. To test antitumor efficacy of dual inhibition of glycolysis and glutaminolysis for renal tumors, Tsc2+/- balb/c litter mates were randomly allocated into seven groups, balanced for gender and of the same age. Tsc2+/- mice were treated for 2 months from the age of 12 months with vehicle (10 μl/kg) (n = 17), 3-BrPA (3 mg/kg) (n = 19), CB-839 (200 mg/kg) (n = 20), rapamycin (4 mg/kg) (n = 20), 3-BrPA (3 mg/kg) plus rapamycin (4 mg/kg) (n = 18), CB-839 (200 mg/kg) plus rapamycin (4 mg/kg) (n = 20), or 3-BrPA (3 mg/kg) plus CB-839 (200 mg/kg) (n = 18). Vehicle or CB-839 was administered twice daily via gavage, and 3-BrPA or rapamycin was administered 5 times a week via intraperitoneal injection. Following treatment, mice were sacrificed for assessment of tumor burden and analysis of protein expression and phosphorylation in the kidneys. Vehicle and CB-839 were supplied by Calithera Biosciences, Inc., South San Francisco, CA. Rapamycin was purchased from LC Laboratories, Woburn, MA, and 3-BrPA was from Sigma-Aldrich, Dorset, UK.

Histology

Assessment of tumor burden in the kidneys of mice was performed as described previously [18]. Mouse kidneys were fixed in 10% buffered formalin saline (Thermo Scientific, Runcorn, UK) for 24 hours. Fixed kidneys were processed and paraffin embedded. Six coronal sections of 5 μm were cut from each kidney. Kidney sections were hematoxylin and eosin (HE)–stained for conventional IHC. Primary antibodies against GAPDH, HK2 and glutamate dehydrogenase (GDH), and phosphorylated S6 ribosomal protein at S235/236, 4E-BP1 at T37/46, and Akt at S473 were supplied by Cell Signalling Technology, Danvers, MA; phosphorylated PKCα at S657 by Santa Cruz Biotechnology Inc., Dallas, TX; GLS, Ki67, and active Caspase-3 by Abcam, Cambridge, UK; and MCT1 by Insight Biotechnology, Wembley, UK. SignalStain Boost Rabbit specific IHC Detection Reagent (Cell Signalling Technology, Danvers, MA) and ImmPACT NovaRED Peroxidase (HRP) Substrate (Vector Laboratories Ltd., Peterborough, UK) were used to stain antigens.

Western Blot

For Western analysis, proteins of kidney tissues were prepared from Tsc2+/- mice treated as described above using AllPrep DNA/RNA/Protein Mini Kit (QiAGEN Ltd-UK, Crawley, UK). Twenty micrograms of protein per sample was separated on NuPAGE 4%–12% Bis-Tris Gels (Fisher Scientific UK Ltd., Loughborough, UK) and transferred onto Hybond ECL Membranes (GE Healthcare UK Ltd., Little Chalfont, UK). Blots were analyzed with ECL Select Western Detection Kit (GE Healthcare UK Ltd.), and signals were detected using Autochemi Imaging System (UVP, Upland, CA). In addition to primary antibodies described above, primary antibodies against β-actin and phosphorylated Akt at T450 were supplied by Cell Signalling Technology, Danvers, MA; phosphorylated mTOR at S2448 and S2481 by Sigma-Aldrich, Dorset, UK; and PKCα at T638 by Abcam, Cambridge, UK. Horseradish peroxidase–conjugated secondary antibody against rabbit was supplied by Cell Signalling Technology, Danvers, MA.

Statistical Analysis

The Mann-Whitney test was performed to compare tumor burden and proliferation of tumor cells between treatment groups. P < .05 was considered to be statistically significant. Analyses were performed using GraphPad Prism 7.03.

Results

Aberrant Activation of mTOR Signaling and Expression of Enzymes Crucial for Glycolysis and Glutaminolysis in Renal Tumors of Tsc2+/- Mice

Tsc2+/- mice spontaneously develop renal lesions including cystic/papillary adenomas and solid carcinomas. As reported previously [19], both mTORC1 and mTORC2 were aberrantly activated in renal tumors of Tsc2+/- mice as indicated by increased phosphorylation of ribosomal protein S6 at S235/236, 4E-BP1 at T37/46, Akt at S473, and PKCα at S657 (Figure 1). GAPDH and HK2 are important glycolytic enzymes. GLS and GDH are enzymes crucial for glutaminolysis. As shown in Figure 2, GAPDH was highly expressed in papillary adenomas and carcinomas. HK2 was less expressed in papillary adenomas, but its expression was increased in carcinomas (Figure 2). The GLS level was variable in papillary adenomas and...
significantly increased in carcinomas (Figure 2). Highly expressed GDH was also observed in papillary adenomas and carcinomas (Figure 2).

**Efficacy of Dual Inhibition of Glycolysis and Glutaminolysis for Therapy of Renal Tumors in Tsc2+/- Mice**

We tested efficacy of dual inhibition of glycolysis and glutaminolysis with 3-BrPA and CB-839 for therapy of renal tumors in Tsc2+/- mice. 3-BrPA is an efficient inhibitor of GAPDH and HK2, and CB-839 is a potent inhibitor of GLS. We first examined the expression of monocarboxylate transporter 1 (MCT1) since it is required for efficient delivery of 3-BrPA to tumor cells [21]. MCT1 was highly expressed in all tumor cells of renal cystic/papillary adenomas and carcinomas in Tsc2+/- mice (Figure 3). We then treated Tsc2+/- mice for 2 months from the age of 12 months as summarized in Table 1. Three mice treated with 3-BrPA alone, three with combination of 3-BrPA and rapamycin, and four with combination of 3-BrPA and CB-839 were killed due to significant loss of body weight within the first month of treatment and excluded from further analysis in this study. Tumor burden was compared by analyzing total number, size, and cellular areas of all lesions (cystic/papillary adenomas and solid carcinomas) and solid carcinomas alone, respectively (Figure 4). When all lesions are analyzed, combination of 3-BrPA and CB-839 significantly reduced overall lesion size ($P = .0209$) and cellular area ($P = .0397$) (Figure 4; Supplementary Table 1). Combination of 3-BrPA and CB-839 also significantly reduced total number ($P < .0001$, $P < .0001$, $P < .0001$), size ($P < .0001$, $P < .0001$, $P = .0054$), and cellular area ($P < .0001$, $P < .0001$, $P < .0001$) of all lesions (Figure 4; Supplementary Table 1). Combination of 3-BrPA and CB-839 also significantly...
reduced total number \((P = .0311)\), size \((P = .0220)\), and cellular area \((P = .0226)\) of solid carcinomas (Figure 4; Supplementary Table 2). Similarly, rapamycin alone or rapamycin plus 3-BrPA or rapamycin plus CB-839 again significantly reduced total number \((P < .0001, P < .0001, P < .0001)\), size \((P < .0001, P < .0001, P < .0001)\), and cellular area \((P < .0001, P < .0001, P < .0001)\) of solid carcinomas (Figure 4; Supplementary Table 2). However, 3-BrPA or CB-839 alone did not significantly reduce total number, size, or cellular area of all lesions or solid carcinomas.

Effect of Treatment on mTOR Signaling and Proliferation in Renal Tumors in Tsc2+/- Mice

We first examined the effect of treatment on mTOR signaling in the kidney tissues by Western blot. We used phosphorylation of S6 ribosomal protein at S235/236 and mTOR at S2448 as readouts for mTORC1 activity and phosphorylation of Akt at S473, Akt at T450,
mTOR at S2481, and PKCα at T638 for mTORC2 activity. As shown in Figure 5a, rapamycin alone or rapamycin plus 3-BrPA or rapamycin plus CB-839 reduced phosphorylation of S6 ribosomal protein, and rapamycin plus 3-BrPA also reduced phosphorylation of mTOR at S2448. Combination of 3-BrPA and CB-839 reduced phosphorylation of S6 ribosomal protein although to a lesser degree (Figure 5a). Rapamycin in combination with 3-BrPA or with CB-839 reduced phosphorylation of Akt at S473 and mTOR at S2481, but

### Table 1. Summary of Animal Treatment

| Treatment Group | Number of Tsc2+/− Mice | Number of Males | Number of Females | Treatment Start Age (Months) | Treatment End Age (Months) | Dosage | Number of Animals Killed Due to Loss of Body Weight Within the First Month of Treatment
|-----------------|------------------------|-----------------|-------------------|-------------------------------|----------------------------|--------|--------------------------------------------------|
| Vehicle         | 17                     | 8               | 9                 | 12                            | 14                         | 10 μl/g | 0                                                |
| 3-BrPA          | 19                     | 9               | 10                | 12                            | 14                         | 3 mg/kg | 3                                                |
| CB-839          | 20                     | 9               | 11                | 12                            | 14                         | 200 mg/kg | 0                                             |
| Rapamycin       | 20                     | 8               | 12                | 12                            | 14                         | 4 mg/kg | 0                                                |
| 3-BrPA+rapamycin| 18                     | 8               | 10                | 12                            | 14                         | 3 mg/kg+4 mg/kg | 3                     |
| CB-839+rapamycin| 20                     | 8               | 12                | 12                            | 14                         | 200 mg/kg+4 mg/kg | 0                     |
| 3-BrPA+CB-839   | 18                     | 8               | 10                | 12                            | 14                         | 3 mg/kg+200 mg/kg | 4                     |

* Tsc2+/− mice were treated twice daily with vehicle or CB-839 via gavage and five times a week with 3-BrPA or rapamycin via intraperitoneal injection.

† These mice were killed due to significant loss of body weight within the first month of treatment.

Figure 4. Efficacy of treatment on renal tumors of Tsc2+/− mice. Tsc2+/− mice were treated from 12 months old for 2 months in 7 groups: vehicle (n = 17), 3-BrPA (n = 19), CB-839 (n = 20), rapamycin (n = 20), 3-BrPA+rapamycin (n = 18), CB-839 + rapamycin (n = 20), and 3-BrPA+CB-839 (n = 18). Three mice from the 3-BrPA group, three mice from the 3-BrPA+rapamycin group, and four mice from the 3-BrPA+CB-839 group were euthanized due to significant loss of body weight within the first month of treatment and excluded from further analysis in this study. Dosages are described in methods. After treatment, kidney sections were prepared for histological assessment of treatment efficacy. Left panel: comparison of total number and size (area) as well as cellular area of all lesions (cystic, papillary, and solid). Right panel: comparison of total number and size (area) as well as cellular area of solid carcinomas. Horizontal bars indicate a median. For detailed statistical analysis, see Supplementary Tables 1 and 2.
rapamycin alone did not (Figure 5a). No consistent changes in phosphorylation of Akt at T450 or PKCα at T638 were observed in the kidney tissues after treatment.

We then assessed effect of treatment on mTOR signaling in renal tumors of Tsc2+/− mice by MS-IHC. Similarly, we used phosphorylation of S6 ribosomal protein at S235/236 as a readout for mTORC1 activity and phosphorylation of Akt at S473 as a readout for mTORC2 activity. As shown in Figure 5b, rapamycin alone or rapamycin plus 3-BrPA or rapamycin plus CB-839 consistently reduced phosphorylation of S6 ribosomal protein in cystic/papillary adenomas and solid carcinomas. Combination of 3-BrPA and CB-839 also slightly reduced phosphorylation of S6 ribosomal protein in cystic/papillary adenomas and solid carcinomas (Figure 5b). Rapamycin plus 3-BrPA or rapamycin plus CB-839 substantially reduced phosphorylation of Akt at S473 in cystic/papillary adenomas and solid carcinomas (Figure 5b). 3-BrPA alone or 3-BrPA plus CB-839 appeared to reduce phosphorylation of Akt at S473 in cystic/papillary adenomas and solid carcinomas although to a lesser degree (Figure 5b). Rapamycin alone reduced phosphorylation of Akt at S473 in solid carcinomas, but a proportion of rapamycin-treated cystic/papillary adenomas showed highly phosphorylated Akt at S473, consistent with previous findings (Figure 5b) [22].

We used Ki67 as a marker to investigate proliferation in renal tumors of Tsc2+/− mice by IHC (Figure 6). Fifteen or more renal tumors from each treatment group were randomly selected for proliferation analysis. Ki67-positive cells were identified using ImageJ, and percentage of Ki67-positive cells in total tumor cells was used for comparison. Representative sections were presented to show expression of Ki67 in tumor cells. Black lines are scale bars. The right bottom panel shows comparison of percentage of Ki67-positive tumor cells between vehicle and other treatment groups (see Supplementary Table 3 for detailed statistical analysis.).

Figure 5. Effect of treatment on mTOR activity in normal tissues and tumors of the kidneys in Tsc2+/− mice. (a) Western blot analysis. Proteins were prepared from normal kidney tissues of 14-month-old Tsc2+/− mice treated as described above. Beta-actin was used as a loading control. Representative Western blots were presented to show phosphorylation of mTOR at S2448 and S2481, S6 at S235/236, 4E-BP1 at T37/46, Akt at T450 and S473, and PKCα at T638. (b) MS-IHC analysis. Kidney sections were prepared from 14-month-old Tsc2+/− mice treated as described above. The same sections were used to stain phosphorylation of S6 at S235/236 and Akt at S473. Representative MS-IHC-stained sections were presented to show phosphorylation of S6 at S235/236 and Akt at S473 in renal tumors. Black lines are scale bars.

Figure 6. Effect of treatment on proliferation of renal tumor cells in Tsc2+/− mice. Kidney sections were prepared from 14-month-old Tsc2+/− mice treated as described above and stained with antibody against Ki67 by IHC to assess proliferation of tumor cells. Fifteen or more renal tumors from each treatment group were randomly selected for proliferation analysis. Ki67-positive cells were identified using ImageJ, and percentage of Ki67-positive cells in total tumor cells was used for comparison. Representative sections were presented to show expression of Ki67 in tumor cells. Black lines are scale bars. The right bottom panel shows comparison of percentage of Ki67-positive tumor cells between vehicle and other treatment groups (see Supplementary Table 3 for detailed statistical analysis.).
tumors from each treatment group were randomly selected for proliferation analysis. Ki67-positive cells were identified using ImageJ, and percentage of Ki67-positive cells in total tumor cells was used for comparison. Rapamycin alone, rapamycin plus 3-BrPA, or rapamycin plus CB-839 substantially reduced the median percentage of Ki67-positive cells from 16.3% to 2.6%, 1.1%, or 0.9%, respectively ($P < .0001$) (Figure 6; Supplementary Table 3). 3-BrPA plus CB-839 also slightly reduced the median percentage of Ki67-positive cells from 16.3% to 13.3% ($P = .0275$) (Figure 6; Supplementary Table 3).

We examined levels of active caspase-3 by IHC to assess apoptosis in renal tumors of $Tsc2^{+/−}$ mice. We did not observe any consistent increase in apoptosis in solid carcinomas following treatment (Supplementary Figure 1). In contrast, reduced levels of active caspase-3 were observed in tumors treated with rapamycin alone, rapamycin plus 3-BrPA, or rapamycin plus CB-839. We also noticed that expression of active caspase-3 was very variable in untreated renal tumors and larger carcinomas appeared to have more tumor cells positive for active caspase-3.

### Discussion

Upregulated aerobic glycolysis and glutaminolysis are required in rapidly proliferating tumor cells to meet increased energy demands and biosynthetic activity [23]. Inhibition of glycolysis or glutaminolysis has been investigated for treatment of various types of cancer in preclinical studies and clinical trials [24,25]. Dual inhibition of glycolysis with 2-deoxy-glucose and glutaminolysis with aminooxycetate effectively suppressed proliferation of ovarian cancer cells *in vitro* [26]. Nonetheless, tumor therapy through dual inhibition of glycolysis and glutaminolysis has hardly been tested *in vivo*. In this study, we studied the efficacy of dual inhibition of glycogenolysis and glutaminolysis for therapy of renal lesions in $Tsc2^{+/−}$ mice. 3-BrPA is an efficient inhibitor of glycolysis by primarily targeting GAPDH and HK2 [11]. MCT1 is an essential transporter required for 3-BrPA-mediated inhibition of glycolysis [21]. CB-839 is a potent blocker of glutaminolysis by selectively inhibiting GLS [13]. We found that GAPDH, HK2, MCT1, and GLS were all highly expressed in renal carcinomas. We showed that combination of 3-BrPA and CB-839 significantly reduced overall tumor burden when renal carcinomas or all renal lesions (cystic/papillary adenomas and carcinomas) were analyzed in the $Tsc2^{+/−}$ mice. These results suggest that the combined treatment may be a better strategy for TSC-associated lesions. However, combination of 3-BrPA and CB-839 was not as efficacious as rapamycin alone or rapamycin in combination with either 3-BrPA or CB-839 for these tumors. Furthermore, significant loss of body weight occurred in some mice treated with 3-BrPA alone, combination of 3-BrPA and rapamycin, or combination of 3-BrPA and CB-839, indicating increased toxicity. Combination of CB-839 and everolimus (a rapamycin analog) has previously showed a greater antitumor activity than everolimus alone in a xenograft mouse model of renal carcinoma and is currently being tested in patients with renal carcinoma [16]. However, we did not find a significant difference in antitumor efficacy between rapamycin alone and rapamycin plus CB-839 in the $Tsc2^{+/−}$ mice. Rapamycin/rapalog alone or CB-839 significantly increased and Akt is activated in $Tsc2^{+/−}$ in vivo. In this study, we examined the effect of treatment on mTOR signaling in normal tissues and tumors from the kidneys of $Tsc2^{+/−}$ mice. Rapamycin alone or rapamycin in combination with either 3-BrPA or CB-839 potently inhibited mTORC1, while combination of 3-BrPA and CB-839 only slightly decreased mTORC1 activity in both normal and tumor tissues. Such mTORC1 inhibition was associated with reduced proliferation of tumor cells, possibly accounting for antitumor activity of these treatments, with rapamycin alone or rapamycin in combination with either 3-BrPA or CB-839 being significantly more effective for these tumors in $Tsc2^{+/−}$ mice. Notably, rapamycin alone or rapamycin in combination with either 3-BrPA or CB-839 was much more efficient in reducing lesion number than combination of 3-BrPA and CB-839, consistent with a role of mTORC2 activity. Feedback inhibition of mTORC2 activity after rapamycin treatment is believed to be one of the reasons for limited efficacy [35]. However, inhibition of mTORC2 may promote or suppress death of tumor cells depending on cellular contexts [36,37]. In addition, no increased apoptosis was observed in renal tumors treated with any single or combined compounds in this study. It remains to be further...
examined whether inhibition of mTORC2 contributes to antitumor efficacy in TSC-associated renal lesions.

We conclude that combination of 3-BrPA and CB-839 may not offer a better therapeutic strategy than rapamycin for TSC-associated tumors, although the combination therapy significantly reduced overall size of all renal lesions.

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**Conflict of Interest Statement**

The authors declare no conflict of interest.

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