Efficacy of Sequential Treatment with Sunitinib-Everolimus in an Orthotopic Mouse Model of Renal Cell Carcinoma

JAMES LARKIN[1], NORBERT ESSER[2*], EMILIANO CALVO[3], ZENTA TSUCHIHASHI[4†], ULRIKE FIEDLER[2*], RALPH GRAESER[2*] and DENNIS KIM[5]

[1]*Department of Medicine, Royal Marsden Hospital, London, U.K.;*
[2]*ProQinase GmbH, Tumor Biology Center Freiburg, Freiburg, Germany;*
[3]*Centro Integral Oncológico Clara Campal, Hospital Madrid Norte-Sanchinarro, Madrid, Spain;*
Novartis Pharmaceuticals Corporation,
[4]*Oncology Biomarkers & Clinical Imaging and* [5]*Oncology Global Medical Affairs, East Hanover, NJ, U.S.A.*

Abstract. *Background/Aim: Sequential treatment with targeted agents is standard of care for patients with metastatic renal cell carcinoma (mRCC). However, clinical data directly comparing treatment outcomes with a mammalian target of rapamycin inhibitor or a vascular endothelial growth factor-targeted agent in the second-line setting are lacking. We evaluated sequential treatment in a syngeneic, orthotopic mouse model of mRCC. Materials and Methods: BALB/c mice were orthotopically implanted with murine RCC (RENCA) cells expressing luciferase and randomized to vehicle, sunitinib, sunitinib followed by sorafenib, or sunitinib followed by everolimus. Tumor growth and metastases were assessed by in vivo (whole body) and ex vivo (primary tumor, lung, liver) luciferase activity and necropsies, performed on day 20 or 46 for vehicle and treatment groups, respectively. Results: Sunitinib followed by everolimus was associated with reduced luciferase activity and primary tumor weight and volume compared with sunitinib, and sunitinib followed by sorafenib. Conclusion: Sequential therapy with sunitinib followed by everolimus demonstrated significant antitumor and anti-metastatic effects.*

*Former employee of ProQinase GmbH.

†Former employee of Novartis Pharmaceuticals Corporation.

Correspondence to: James Larkin, FRCP, Ph.D., Department of Medicine, Royal Marsden Hospital, Fulham Road, London SW3 6JJ, U.K. Tel: +44 207 808 2198, Fax: +44 207 808 2688, e-mail: james.larkin@rmh.nhs.uk

Key Words: Genitourinary cancers, metastasis, metastasis genes, metastasis models, animal models of cancer, cellular responses to anticancer drugs, reversal of drug resistance, kinase and phosphatase inhibitors, agents with other mechanisms of action.

Optimal sequencing of targeted therapies in patients with metastatic renal cell carcinoma (mRCC) is a key clinical issue. Currently approved agents target two distinct molecular pathways, the vascular endothelial growth factor receptor (VEGFR) and the mammalian target of rapamycin (mTOR) pathways. Sunitinib is a VEGFR-tyrosine kinase inhibitor (TKI) with a category 1 recommendation for use as a first-line therapy in mRCC (1). Other recommended first-line therapies include pazopanib, bevacizumab plus interferon-alpha, and temsirolimus (for patients with poor prognosis) (1). In the second-line setting, the VEGFR-TKIs sorafenib, sunitinib, and pazopanib have a category 1 recommendation for the treatment of patients who have received prior cytokine therapy (1). Everolimus is an mTOR inhibitor with a category 1 recommendation for use as a second-line treatment of mRCC after failure of initial VEGFR-TKI therapy (1). This recommendation is based on results of the phase III RECORD-1 study, which demonstrated that everolimus significantly improved progression-free survival (PFS) compared with placebo [median PFS=4.9 months *versus* 1.9 months, respectively; hazard ratio (HR)=0.33; $p<0.001$] in patients with mRCC who had failed previous VEGFR-TKI therapy (2).

The direct combination of two targeted agents has shown unacceptable toxicity in several trials of patients with mRCC (3-5); thus, sequential treatment has emerged as the standard of care in current clinical practice. Several options are available for patients who fail initial VEGFR-TKI therapy, including treatment with an mTOR inhibitor or treatment with a second VEGFR-TKI. Efficacy observed in prospective clinical studies in patients with VEGFR-TKI-refractory disease suggests at least partial cross-resistance to second-line VEGFR-TKI therapy (6, 7). For example, in the phase III AXIS trial of axitinib *versus* sorafenib in patients who had failed one previous anticancer therapy, median PFS with axitinib was 12.1 months in patients previously treated with

cytokines and 4.8 months in patients previously treated with sunitinib (7). High incidences of certain class-effect toxicities in patients treated with sequential VEGF-targeted therapies have also been reported (6, 8). Additionally, recent preclinical data suggest that although VEGFR-TKIs inhibit primary tumor growth, they may also cause tumors to progress to more metastatic and invasive phenotypes, leading to enhanced levels of malignancy and reduced overall survival (9-11). This potential for cross-resistance, cumulative toxicity, and increased invasiveness suggests that switching to a second-line treatment with a distinctly different mechanism of action, such as mTOR inhibition, may be a beneficial strategy for overcoming VEGFR-TKI resistance (12).

While sequential treatment with targeted agents has become the standard of care in patients with mRCC, no head-to-head clinical studies have been conducted to date that directly compare the safety and efficacy of an mTOR inhibitor and a VEGFR-TKI in patients who have failed initial VEGFR-TKI therapy. The effectiveness of this strategy also has yet to be evaluated in a preclinical setting. Thus, the aim of the present study was to explore the outcomes of sequential VEGFR-TKI followed by VEGFR-TKI (*i.e.* sunitinib followed by sorafenib) and VEGFR-TKI followed by mTOR inhibitor (*i.e.* sunitinib followed by everolimus) therapy in a syngeneic, orthotopically implanted mouse model of mRCC, and, in particular, whether these different therapeutic sequences led to distinct effects on metastases or primary tumors.

Materials and Methods

Cells and culture conditions. The murine RENCA cell line, originally obtained from a tumor that arose spontaneously in the kidney of a BALB/c mouse, is a well-characterized model for human RCC (13). RENCA cells were obtained from the ATCC® (LGC Standards GmbH, Wesel, Germany) (CRL-2947™ ; of murine origin as confirmed by genomic fingerprinting). Murine origin was further confirmed *via* genotyping by the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures on August 18, 2009. The cells were transduced using a retrovirus expressing a luciferase-neomycin (LN) fusion protein (14). Stably transduced RENCA-LN cell pools were selected using 1 mg/ml of G418-sulphate (#P11-012, PAA Laboratories GmbH, Pasching, Austria). RENCA-LN cells were grown as monolayers in DMEM and phenol red supplemented with 10% fetal calf serum, 2 mM of L-glutamine, 100 units of penicillin/ml, and 100 μg of streptomycin/ml and then cultured at 37˚C in a humidified atmosphere (90% air, 10% carbon dioxide). Cells were routinely split every three days at a ratio of 1:5 to 1:10 using trypsin/EDTA and were seeded at approximately 1 to 2×10^6 cells/80 cm^2 in 10-ml aliquots of medium.

In vivo experiments. Female BALB/c mice (Charles River GmbH, Sulzfeld, Germany) approximately six weeks old and weighing approximately 20 g were used for *in vivo* studies. All mice were anesthetized on day 0 with 1.5 to 2.0 v/% isoflurane in combination with an oxygen flow of 2 l/min and orthotopically implanted with 4×10^5 RENCA-LN cells in 25 μl phosphate-buffered saline (PBS) into the left kidney *via* subcapsular injection. Growth of the resultant RENCA-LN tumors and metastases were monitored by *in vivo* bioluminescence imaging. All procedures were approved by the Ethics Committee for Animal Experimentation according to the United Kingdom Coordinating Committee on Cancer Research Guidelines (15).

Treatment groups. Tumor-bearing mice were randomized to one of four treatment groups (groups A to D, 12 animals per group) according to their luciferase signal on day 3. Group A received 10 ml/kg vehicle (PBS) once daily on days 4 to 20; group B received 40 mg/kg sunitinib on days 4 to 46; group C received 40 mg/kg sunitinib once daily on days 4 to 34 followed by 30 mg/kg sorafenib once daily on days 35 to 46; and group D received 40 mg/kg sunitinib once daily on days 4 to 34 followed by 10 mg/kg everolimus on days 35 to 46. All treatments were administered orally in volumes of 200 μl per 20 g body weight. Doses of sunitinib, sorafenib, and everolimus were chosen based on previous literature reports of efficacy in mouse models of RCC (16-18), as well as internal data. The treatment was switched when the average bioluminescence signal of animals in group C reached the half maximum signal of the control group (group A).

Evaluation of tumors and metastases via bioluminescence imaging. Tumor growth and the occurrence of metastases were monitored on days 3, 6, 13, 20, 27, 34, 40, and 45 using *in vivo* bioluminescence imaging. Mice were anesthetized and administered 2 mg D-luciferin intraperitoneally as two 50-μl volumes. Light emission levels were measured 10 min after injection and captured by a CCD camera for 1 to 5 min using the NightOWL LB 981 bioluminescence imaging system (Berthold Technologies, Germany). Animals were killed by cervical dislocation on day 20 due to ethical reasons (group A), or day 46 (groups B, C, D) and necropsies were performed. Primary tumor wet weight and volume, lung weight, and numbers of lung metastases were determined. Primary tumor tissues were collected and divided into two parts: one section of each tumor was snap-frozen in liquid nitrogen and stored at −80˚C and the other was analyzed using an *ex vivo* bioluminescence luciferase assay (E1501, Promega). The lung, liver, splenic, and ileal tissues were also collected, homogenized, and analyzed for metastases according to *ex vivo* luciferase activity. With the exception of the primary tumor, protein concentrations determined by the Bradford assay were used to normalize the luciferase activities (14). Blood samples were taken from each animal *via* the retro-orbital vein plexus and directly collected into EDTA tubes and centrifuged for 10 min at 5000× *g* at 4˚C. The supernatant (EDTA plasma) was stored at −20˚C.

Statistical analysis. The data were expressed as the means±SEM and statistical analyses of efficacy data were performed using the Mann-Whitney test. *p*-Values of the unpaired *t*-test were additionally determined when required.

Results

In order to evaluate the effects of sequential therapy in a preclinical model of RCC, a total of 48 female BALB/c mice were implanted orthotopically with luciferase-marked RENCA cells, and randomized to four groups according to whole-body bioluminescence imaging. Group A served as an untreated control, whereas groups B-D received sunitinib at

a daily dose of 40 mg/kg. Treatment effects on tumor and metastatic burden were monitored once weekly using bioluminescence imaging. Animals from the untreated control group were sacrificed after 20 days for ethical reasons. Animals from groups B to D were treated with sunitinib until the mean tumor size in each group reached 50% that of the control group at day 20, according to bioluminescence measurements. This was observed at day 34 for all groups. Group B then continued to receive sunitinib, whereas groups C and D received sorafenib (30 mg/kg qd) or everolimus (10 mg/kg qd), respectively. After two weeks, all animals in groups B to D were killed, and tumor size as well as metastasis burden in potential target organs were measured.

Treatment-induced toxicity. Two animals from group B and two animals from group C were killed early (day 31). One animal from group D was found dead on day 38. Overall body weights remained stable across all treatment groups during the course of the study. Mice in the vehicle control arm (group A) demonstrated a slight decrease in body weight (5.8%) between days 7 and 14 that stabilized thereafter.

In vivo luciferase activity. The *in vivo* bioluminescence signal determined by whole-body imaging (both primary tumors and metastases) was assessed weekly during the study. Animals in group A (vehicle control) showed a sustained increase in luciferase activity, and by implication tumor volume, from days 4 to 20 before they were killed on day 20 (Figure 1). The mean *in vivo* bioluminescence signal for animals in groups B, C, and D, which were treated with sunitinib between days 4 and 34, increased over time but at a lower rate than that of the vehicle-treated group. In animals that continued treatment with sunitinib (group B) or switched from sunitinib to sorafenib (group C), mean *in vivo* luciferase activity stabilized from days 35 to 46. In animals that were switched to everolimus treatment (group D), a notable decrease in mean *in vivo* luciferase signaling was observed, suggesting a reduction in tumor volume. The reduction in the mean *in vivo* luciferase activity in animals in group D was significantly greater compared with both groups B (p=0.0022) and C (p=0.0054) according to the Mann-Whitney method.

Primary tumors: necropsy findings and ex vivo luciferase activity. Animals of group A (vehicle control) were killed on day 20, with all other groups terminated on day 46. Primary tumor volumes and wet weights (mean±SEM) were determined during necropsy for each group. Primary tumor volumes were 2.59 (±0.33), 1.86 (±0.26), 2.87 (±0.51), and 0.77 (±0.11) cm^3 in groups A to D, respectively (Figure 2A). Treatment of animals with a sunitinib to everolimus sequence (group D) resulted in a significantly lower primary tumor volume compared with animals that remained on sunitinib (group B) or were switched to sorafenib (group C) (p<0.001 for both comparisons). Evaluation of primary tumor wet weights showed a similar pattern to the tumor volume measurements and were 2.56 (±0.28), 1.63 (±0.20), 2.31 (±0.21), and 0.66 (±0.09) g in groups A to D, respectively (Figure 2B). The tumor wet weights in animals treated with everolimus were significantly lower than those of the other treatment groups (Mann-Whitney test: p<0.001).

Luciferase activity in the primary tumor, which is a function of the number of live tumor cells, was measured using an *ex vivo* luciferase assay. The results correlated well with the tumor size and weight measurements, confirming that treatment with sunitinib followed by everolimus (group D) resulted in a significant reduction of the number of viable tumor cells when compared with sunitinib followed by sorafenib (group C; p=0.0001) or sunitinib alone (group B; p=0.0001) (Figure 2C).

Metastases in select target organs: necropsy findings and ex vivo luciferase activity. The quantities and weights of lung metastases were also assessed in all animals. The mean numbers of lung metastases were 65.75 (±62.22), 46.80 (±13.16), 66.00 (±18.68), and 23.64 (±3.54) in groups A to D, respectively (Figure 3A). The quantification of metastases in animals with a high metastatic burden in the lung is likely to be underestimated as metastatic growth adopted a state of confluency which indicated collation of multiple metastases. Lung wet weights were also evaluated and were 0.14 (±0.01), 0.23 (±0.06), 0.50 (±0.12), and 0.17 (±0.01) g in groups A to D, respectively (Figure 3B). According to the Mann-Whitney test, treatment with sunitinib alone (group B) significantly increased total lung weight compared with vehicle (group A; p=0.0053), while the sequential addition of sorafenib (group C; p=0.0440) appeared to further potentiate this increase. The mean lung weights were significantly lower in the sunitinib followed by everolimus-treated group (group D) compared with the sunitinib followed by sorafenib-treated group (group C) (p=0.044).

Luciferase activity, which served as a means of quantifying the number of tumor cells spread into other organs, was determined from extracts derived from lung, liver, spleen, and ileum as potential target organs (Figure 4). Treatment of animals with sequential sunitinib and everolimus also yielded significantly lower *ex vivo* luciferase activity in lung and liver (p<0.005), and spleen and ileum (p<0.05) compared with sequential sunitinib and sorafenib. Lung and liver *ex vivo* luciferase activity was also significantly lower in animals receiving sunitinib followed by everolimus compared with those receiving sunitinib alone (p<0.05), while the observed differences in splenic and ileal luciferase activity between these groups failed to achieve statistical significance because of the small number of metastases in all groups.

Discussion

The results of this study suggest that sequential administration of sunitinib followed by everolimus is associated with significant *in vivo* antitumor and anti-metastatic activity. Switching therapy from sunitinib to everolimus reduced both primary tumor and metastatic burden, with almost complete clearance of tumor cells, in this clinically relevant, orthotopic syngeneic mouse model of RCC. RENCA is a robust and well-established model of RCC, offering the advantage of rapid and extensive tumor growth within the primary tissue of origin and creating an organ-specific environment for the development of early metastases (13, 19). Furthermore, the use of immunologically intact mice enables consideration of effects on the antitumor immune response and, thus affords a more realistic tumor micro-environment than alternative, orthotopic models of RCC (19). Nevertheless, our experiments were conducted in a single RCC cell line and results remain to be confirmed in additional models.

In the overall assessment of tumor burden *in vivo* by bioluminescence, sequential treatment with sunitinib followed by everolimus showed a significant reduction in luciferase activity at day 46 compared with continuous sunitinib and sequential sunitinib followed by sorafenib. Continuation of the study and collection of further data points were not possible because of an ethical requirement to kill sorafenib-treated mice at the time their tumors reached a prescribed limit. However, this single bioluminescence data point was indeed further corroborated by both necropsy and *ex vivo* data.

Quantification by *ex vivo* luciferase activity demonstrated everolimus-treated mice to have reduced activity and, by implication, fewer metastases, in lung, liver, splenic, and ileal tissues. This is compared with the regimens of continuous sunitinib and sequential sunitinib followed by sorafenib, which in this model afforded significantly larger primary tumor weights and volumes with increased metastatic burden. Given the difficulties of accurately quantifying metastases in mice with high tumor burden due to confluency, these *ex vivo* luciferase assays provided a means of corroborating results obtained by necropsy, with consistencies being observed between the necropsy and *ex vivo* results in lung. While direct comparisons between the control arm and the three treatment arms cannot be made because of the differences in necropsy time points (untreated mice had only 20 days to develop metastases, while treated animals had 46 days), comparisons between treatment arms are possible, with significantly greater lung, liver, splenic, and ileal metastatic burden observed in mice administered sequential sunitinib followed by sorafenib compared with sunitinib followed by everolimus, as assessed by necropsy and *ex vivo* luciferase activity.

Inhibitors of the mTOR and VEGF signaling pathways represent the two mechanistic classes of targeted therapies currently approved for the treatment of mRCC. The mTOR pathway is essential for protein synthesis and cell cycle progression, and blockade of mTOR signaling with an mTOR inhibitor has been shown to directly impede tumor cell growth (20), whereas VEGFR-TKIs block VEGF pathway signaling by inhibiting phosphorylation of VEGFR, thereby inhibiting angiogenesis (21). The anti-VEGF antibody bevacizumab sequesters VEGF ligand and prevents downstream signaling to the VEGFR (22). However, the effect of these agents on angiogenesis has been demonstrated to be incomplete and impermanent, with cessation leading to the persistence of the basement membrane and pericytes, allowing for rapid recovery of the vasculature (23). Preclinical experiments have also shown that VEGFR-TKI treatment can cause tumors to progress to a more metastatic and invasive phenotype due to localized hypoxia, leading to greater levels of malignancy and reduced overall survival (9, 10). Data from the current study appear to support this observation, with both continuous sunitinib and sequential sunitinib followed by sorafenib leading to increased metastatic burden compared with sunitinib followed by everolimus.

Recent mechanistic studies assessing the overlapping effects of mTOR and VEGF inhibition on endothelial cell biology and angiogenic processes have suggested that the mTOR pathway may play an integral role in VEGFR-modulated proliferative pathways (24, 25). The VEGFR-TKI sunitinib has been shown to act primarily on tumor endothelium rather than tumors cells (26), whereas everolimus has been shown to have increased activity against more mature vasculature of tumors (24). Preclinical data suggest that combining both mechanisms of action leads to more complete inhibition of tumor vascularization, both

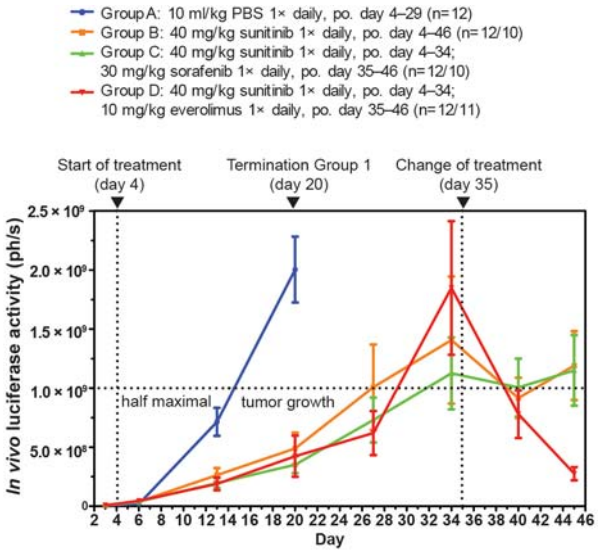

Figure 1. *In vivo luciferase activity of primary tumors and metastases. po: Per os.*

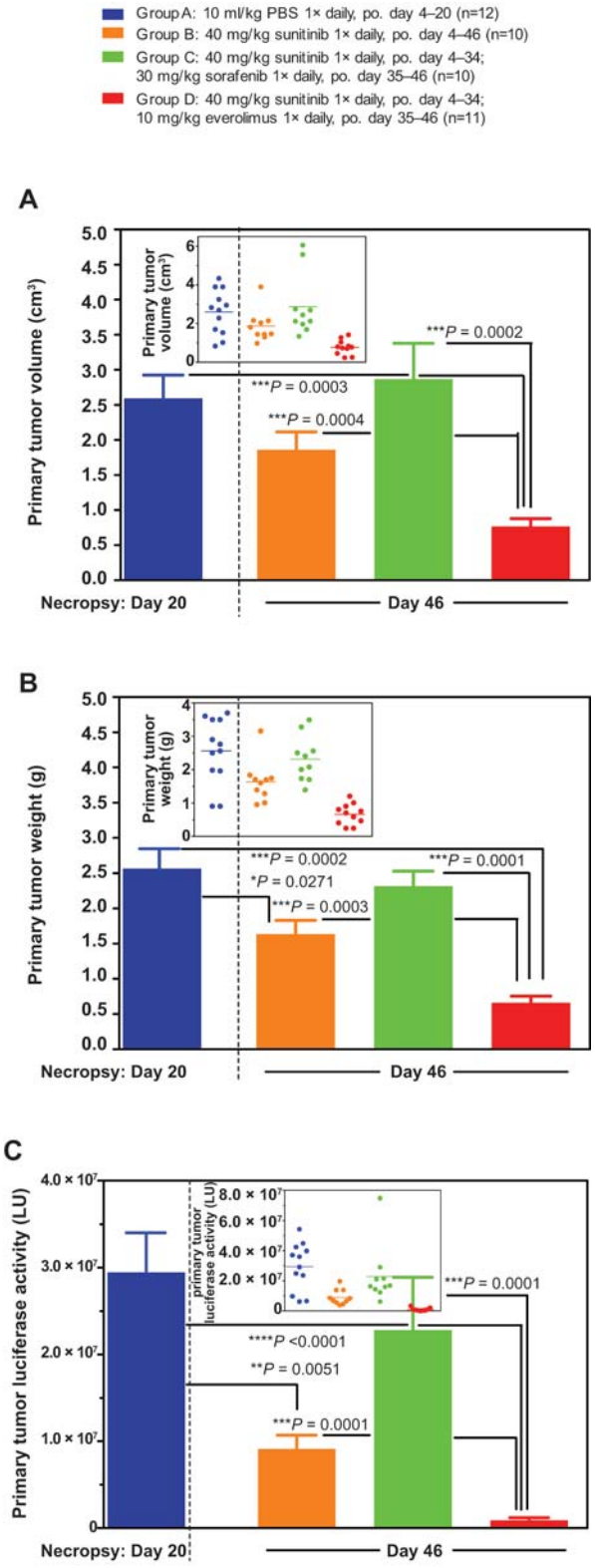

Figure 2. *Primary tumor volume (A), wet weight (B), and ex vivo luciferase activity (C). The insets show the individual data points together with their corresponding mean values. po: Per os.*

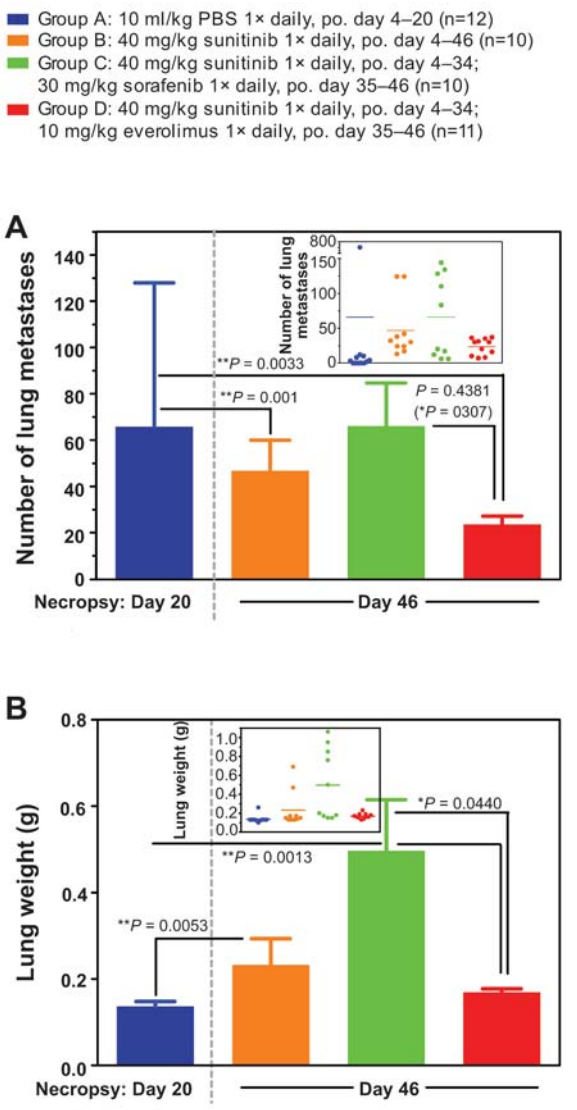

Figure 3. *Number of lung metastases (A) and lung weight (B). The insets show the individual data points together with their corresponding mean values.*

directly (endothelial and smooth muscle cells and pericytes) and indirectly (via VEGF) (24). In a xenograft model of gastric cancer, mTOR inhibition by everolimus was shown to counteract VEGF induction by sunitinib, leading to a significant reduction in tumor burden with long-lasting tumor growth control (25). The antitumor activity of sunitinib in combination with everolimus was also shown to be superior to that of either agent alone (25). In our preclinical model of RCC, the complementary anticancer effects of sunitinib and everolimus, when dosed in sequence, were superior to those of sunitinib alone and sunitinib followed by sorafenib, with respect to both primary tumor growth and metastases.

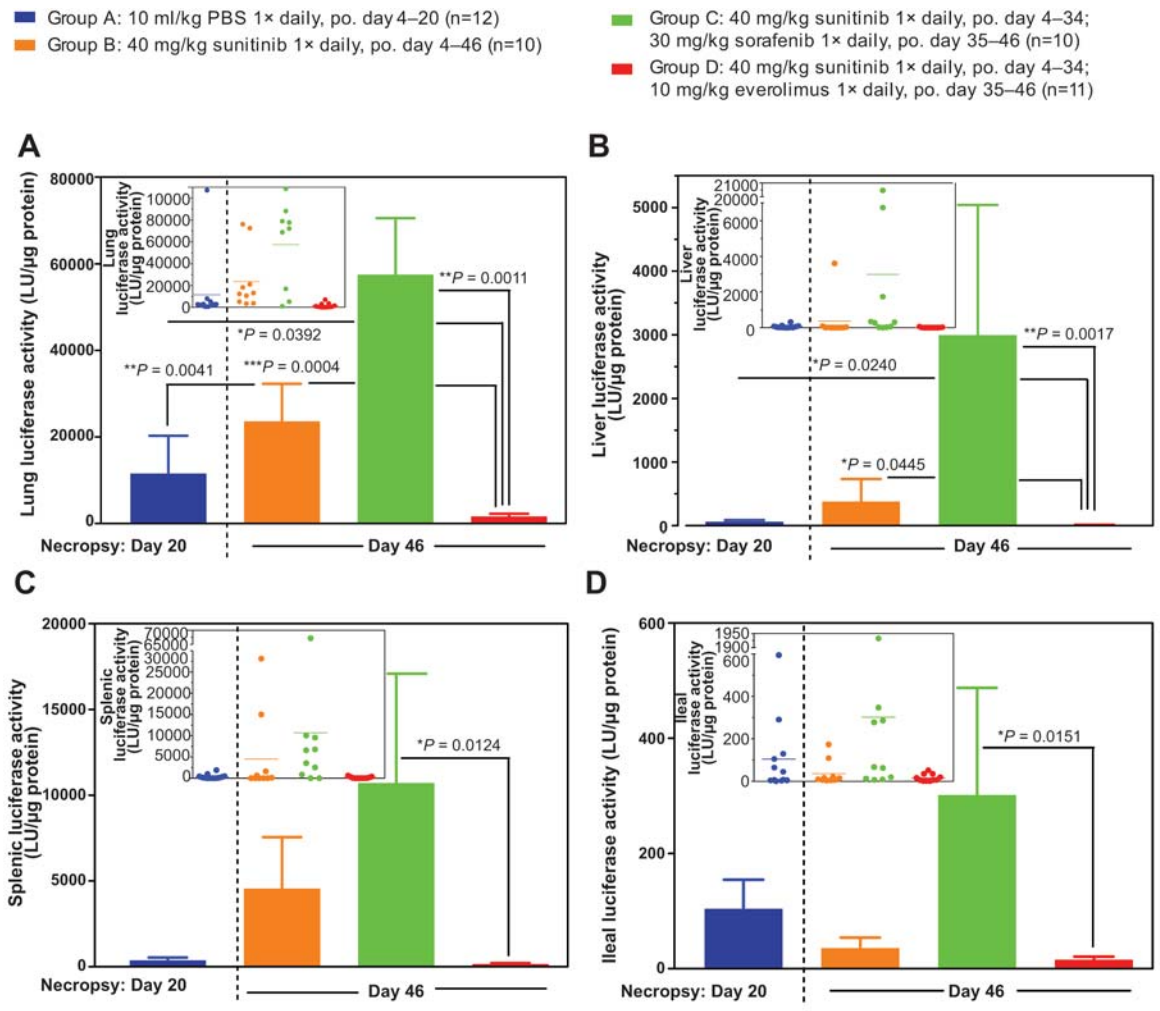

Figure 4. *Ex vivo luciferase activity of lung (A), liver (B), spleen (C), and ileum (D) protein extracts. The insets show the individual data points together with their corresponding mean values. po: Per os.*

The activity of sequential sunitinib followed by everolimus observed in this preclinical study may be reflective of clinical benefit observed with everolimus in sunitinib-refractory patients with mRCC (2, 27, 28). While direct combination of sunitinib and everolimus in patients with mRCC has shown unacceptable toxicity in a phase I trial (3), sequential administration of sunitinib and everolimus is an effective treatment strategy with acceptable safety (2, 27, 28). Everolimus is the recommended standard of care for patients with mRCC who have failed initial VEGFR-TKI therapy (1). Switching the mechanism of action to an mTOR inhibitor following VEGFR-TKI failure may reduce the risk of cumulative class-effect toxicities that have been associated with consecutive VEGFr-TKI therapy (6, 8), and the distinct roles of the VEGF and mTOR pathways in driving tumor growth may lead to synergy when a VEGFR-TKI and an mTOR inhibitor are administered in combination (25), or in sequence. While the dosages of sunitinib, sorafenib, and everolimus employed in our study were carefully selected based on literature precedent and previous experience, it should be noted that the relevance of these to the approved clinical dosages in humans is difficult to ascertain. Nevertheless, our results highlight important potential benefits that may be derived from sequential VEGFR-TKI followed by mTOR inhibitor therapy in patients with mRCC.

In conclusion, sequential treatment with sunitinib followed by everolimus in an orthotopic, syngeneic mouse model of RCC led to significant reductions in both primary tumor and metastatic burden; these effects were not observed with sunitinib alone or sequential therapy of sunitinib followed by sorafenib. These results support the use of everolimus as an integral part of therapy sequencing in mRCC.

Funding Statement

Funding was provided by Novartis Pharmaceuticals.

Conflict of Interest

J. Larkin has served in a consultant/ advisory role for Novartis, Pfizer, Bayer, AVEO, and GlaxoSmithKline, and has received research funding from Novartis and Pfizer. E. Calvo has served in a consultant/ advisory role for Novartis and Pfizer, and has received honoraria and research funding from Novartis and Pfizer. D. Kim and Z. Tsuchihashi are current/past employees of Novartis.

Acknowledgements

Editorial assistance in the preparation of this manuscript was provided by ApotheCom (Yardley, PA, USA) and funded by Novartis Pharmaceuticals.

References

1 National Comprehensive Cancer Network (NCCN). The NCCN Clinical Practice Guidelines in Oncology™ for Kidney Cancer V.1.2012. www.nccn/org. Accessed February 10, 2012.
2 Motzer RJ, Escudier B, Oudard S, Hutson TE, Porta C, Bracarda S, Grünwald V, Thompson JA, Figlin RA, Hollaender N, Kay A, Ravaud A; RECORD-1 Study Group: Phase 3 trial of everolimus for metastatic renal cell carcinoma: final results and analysis of prognostic factors. Cancer *116*: 4256-4265, 2010.
3 Molina AM, Feldman DR, Voss MH, Ginsberg MS, Baum MS, Brocks DR, Fischer PM, Trinos MJ, Patil S and Motzer RJ: Phase 1 trial of everolimus plus sunitinib in patients with metastatic renal cell carcinoma. Cancer *118*: 1868-1876, 2012.
4 Negrier S, Gravis G, Pérol D, Chevreau C, Delva R, Bay JO, Blanc E, Ferlay C, Geoffrois L, Rolland F, Legouffe E, Sevin E, Laguerre B and Escudier B: Temsirolimus and bevacizumab, or sunitinib, or interferon alfa and bevacizumab for patients with advanced renal cell carcinoma (TORAVA): a randomised phase 2 trial. Lancet Oncol *12*: 673-680, 2011.
5 Feldman DR, Baum MS, Ginsberg MS, Hassoun H, Flombaum CD, Velasco S, Fischer P, Ronnen E, Ishill N, Patil S and Motzer RJ: Phase I trial of bevacizumab plus escalated doses of sunitinib in patients with metastatic renal cell carcinoma. J Clin Oncol *27*: 1432-1439, 2009.
6 Di Lorenzo G, Carteni G, Autorino R, Bruni G, Tudini M, Rizzo M, Aieta M, Gonnella A, Rescigno P, Perdonà S, Giannarini G, Pignata S, Longo N, Palmieri G, Imbimbo C, De Laurentiis M, Mirone V, Ficorella C and De Placido S: Phase II study of sorafenib in patients with sunitinib-refractory metastatic renal cell cancer. J Clin Oncol *27*: 4469-4474, 2009.
7 Rini BI, Escudier B, Tomczak P, Kaprin A, Szczylik C, Hutson TE, Michaelson MD, Gorbunova VA, Gore ME, Rusakov IG, Negrier S, Ou YC, Castellano D, Lim HY, Uemura H, Tarazi J, Cella D, Chen C, Rosbrook B, Kim S and Motzer RJ: Comparative effectiveness of axitinib *versus* sorafenib in advanced renal cell carcinoma (AXIS): a randomised phase 3 trial. Lancet *378*: 1931-1939, 2011.
8 Garcia JA, Hutson TE, Elson P, Cowey CL, Gilligan T, Nemec C, Dreicer R, Bukowski RM and Rini BI: Sorafenib in patients with metastatic renal cell carcinoma refractory to either sunitinib or bevacizumab. Cancer *116*: 5383-5390, 2010.
9 Ebos JM, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG and Kerbel RS: Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. Cancer Cell *15*: 232-239, 2009.
10 Paez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, Viñals F, Inoue M, Bergers G, Hanahan D and Casanovas O: Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. Cancer Cell *15*: 220-231, 2009.
11 Hammers HJ, Verheul HM, Salumbides B, Sharma R, Rudek M, Jaspers J, Shah P, Ellis L, Shen L, Paesante S, Dykema K, Furge K, Teh BT, Netto G and Pili R: Reversible epithelial to mesenchymal transition and acquired resistance to sunitinib in patients with renal cell carcinoma: evidence from a xenograft study. Mol Cancer Ther *9*: 1525-1535, 2010.
12 Larkin J, Swanton C and Pickering L: Optimizing treatment of metastatic renal cell carcinoma by changing mechanism of action. Expert Rev Anticancer Ther *11*: 639-649, 2011.
13 Murphy GP and Hrushesky WJ: A murine renal cell carcinoma. J Natl Cancer Inst *50*: 1013-1025, 1973.
14 Bornmann C, Graeser R, Esser N, Ziroli V, Jantscheff P, Keck T, Unger C, Hopt UT, Adam U, Schaechtele C, Massing U and von Dobschuetz E: A new liposomal formulation of gemcitabine is active in an orthotopic mouse model of pancreatic cancer accessible to bioluminescence imaging. Cancer Chemother Pharmacol *61*: 395-405, 2008.
15 Workman P, Balmain A, Hickman JA, McNally NJ, Rohas AM, Mitchison NA, Pierrepoint CG, Raymond R, Rowlatt C, Stephens TC and Wallace J: UKCCCR guidelines for the welfare of animals in experimental neoplasia. Br J Cancer *58*: 109-113, 1988.
16 Rini BI: Sunitinib. Exp Opin Pharmacother *8*: 2359-2369, 2007.
17 Chang YS, Adnane J, Trail PA, Levy J, Henderson A, Xue D, Bortolon E, Ichetovkin M, Chen C, McNabola A, Wilkie D, Carter CA, Taylor IC, Lynch M and Wilhelm S: Sorafenib (BAY 43-9006) inhibits tumor growth and vascularization and induces tumor apoptosis and hypoxia in RCC xenograft models. Cancer Chemother Pharmacol *59*: 561-574, 2007.
18 O'Reilly T and McSheehy PM: Biomarker development for the clinical activity of the mTOR inhibitor everolimus (RAD001): processes, limitations, and further proposals. Translational Oncol *3*: 65-79, 2010.
19 Killion JJ, Radinsky R and Fidler IJ: Orthotopic models are necessary to predict therapy of transplantable tumors in mice. Cancer Metastasis Rev *17*: 279-284, 1998.
20 Morgensztern D and McLeod HL: PI3K/Akt/mTOR pathway as a target for cancer therapy. Anticancer Drugs *16*: 797-803, 2005.
21 Rini BI: Vascular endothelial growth factor-targeted therapy in metastatic renal cell carcinoma. Cancer *115*: 2306-2312, 2009.
22 Ranieri G, Patruno R, Ruggieri E, Montemurro S, Valerio P and Ribatti D: Vascular endothelial growth factor (VEGF) as a target of bevacizumab in cancer: From the biology to the clinic. Curr Med Chem *13*: 1845-1857, 2006.
23 Mancuso MR, Davis R, Norberg SM, O'Brien S, Sennino B, Nakahara T, Yao VJ, Inai T, Brooks P, Freimark B, Shalinsky DR, Hu-Lowe DD and McDonald DM: Rapid vascular regrowth in tumors after reversal of VEGF inhibition. J Clin Invest *116*: 2610-2621, 2006.

24 Lane HA, Wood JM, McSheehy PM, Allegrini PR, Boulay A, Brueggen J, Littlewood-Evans A, Maira SM, Martiny-Baron G, Schnell CR, Sini P and O'Reilly T: mTOR inhibitor RAD001 (everolimus) has antiangiogenic/vascular properties distinct from a VEGFR tyrosine kinase inhibitor. Clin Cancer Res *15*: 1612-1622, 2009.

25 Fuereder T, Jaeger-Lansky A, Hoeflmayer D, Preusser M, Strommer S, Cejka D, Koehrer S, Crevenna R and Wacheck V: mTOR inhibition by everolimus counteracts VEGF induction by sunitinib and improves anti-tumor activity against gastric cancer *in vivo*. Cancer Lett *296*: 249-256, 2010.

26 Huang D, Ding Y, Li Y, Luo WM, Zhang ZF, Snider J, Vandenbeldt K, Qian CN and Teh BT: Sunitinib acts primarily on tumor endothelium rather than tumor cells to inhibit the growth of renal cell carcinoma. Cancer Res *70*: 1053-1062, 2010.

27 Grünwald V, Karakiewics PI, Bavbek SE, Miller K, Machiels JP, Lee SH, Larkin J, Bono P, Rha SY, Castellano D, Blank CU, Knox JJ, Hawkins R, Anak O, Rosamilia M, Booth J, Pirotta N, Bodrogi I; REACT Study Group: An international expanded-access programme of everolimus: Addressing safety and efficacy in patients with metastatic renal cell carcinoma who progress after initial vascular endothelial growth factor receptor-tyrosine kinase inhibitor therapy. Eur J Cancer *48*: 324-332, 2012.

28 Calvo E, Escudier B, Motzer RJ, Oudard S, Hutson TE, Porta C, Bracarda S, Grünwald V, Thompson JA, Ravaud A, Kim D, Panneerselvam A, Anak O and Figlin RA: Everolimus in metastatic renal cell carcinoma: Subgroup analysis of patients with 1 or 2 previous vascular endothelial growth factor receptor-tyrosine kinase inhibitor therapies enrolled in the phase III RECORD-1 study. Eur J Cancer *48*: 333-339, 2012.

Received April 17, 2012
Revised June 10, 2012
Accepted June 11, 2012