RESEARCH PAPER

Continuous administration of the mTORC1 inhibitor everolimus induces tolerance and decreases autophagy in mice

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Received 5 April 2016; **Revised** 31 August 2016; **Accepted** 5 September 2016

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BACKGROUND AND PURPOSE

Everolimus is an allosteric inhibitor of the mechanistic target of rapamycin complex 1 (mTORC1) widely known for its potent autophagy stimulating properties. Because everolimus shows poor solubility and stability in aqueous solutions, long-term *in vivo* administration in preclinical models is challenging. The aim of the present study was to evaluate the effects of short-term and long-term everolimus administration on mTORC1 inhibition and autophagy induction in mice.

EXPERIMENTAL APPROACH

We developed a vehicle in which everolimus was solubilized and stable at 37°C for at least 1 month. Using osmotic minipumps, GFP microtubule-associated protein light chain 3 transgenic mice were treated continuously either with vehicle or everolimus (1.5 mg·kg⁻¹ per day) for 3 or 28 days. Alternatively, a regimen consisting of intermittent everolimus administration (every other day) for 56 days by oral gavage was used. Autophagy markers and mTORC1 activation status were investigated in the liver.

KEY RESULTS

As expected, everolimus inhibited mTORC1 and stimulated autophagy in the liver after 3 days of treatment. However, continuous administration for 28 days resulted in hyperactivation of the Akt1-mTORC1 pathway accompanied by a remarkable decrease in autophagy markers. Everolimus given intermittently for 56 days partially rescued mTORC1 sensitivity to the drug but without inducing autophagy. The failure to induce autophagy following long-term everolimus administration was due to uncoupling of the mTORC1 substrate unc-51 like autophagy activating kinase 1.

CONCLUSIONS AND IMPLICATIONS

Our data encourage the use of intermittent everolimus regimens to prevent tolerance and to extend its activity.

Abbreviations

ACTB, β -actin; LC3, microtubule-associated protein light chain 3; mTORC1/2, mechanistic target of rapamycin, complex 1/2; RCC, renal cell carcinoma; p70S6K, ribosomal protein S6 kinase B1; S6rp, S6 ribosomal protein; SQSTM1, sequestosome 1; ULK1, unc-51 like autophagy activating kinase

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These Tables list key protein targets and ligands in this article that are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015).

Introduction

Everolimus (RAD001) is one of several semisynthetic derivatives of rapamycin, collectively known as rapalogs, with superior pharmacokinetics and pharmacodynamics as compared with the parent compound (Maurizio Salvadori, 2011; Lamming *et al.*, 2013). The drug is widely used in immunosuppressive and cancer therapy as well as in drug-eluting stents (Wander *et al.*, 2011; De Meyer *et al.*, 2015). Like all rapalogs, everolimus binds to its intracellular target FK-506 binding protein 1A to form a complex that inhibits the mechanistic target of rapamycin (mTOR) in an allosteric manner (Hay and Sonenberg, 2004; Dowling *et al.*, 2010). mTOR is a large (~270 kDa) serine/threonine kinase highly conserved in all eukaryotes that can be found in two distinct complexes: mTOR complex 1 (mTORC1) and mTORC2. Rapalogs are mainly mTORC1 inhibitors (Martinet *et al.*, 2014a; Kim and Guan, 2015; Kurdi *et al.*, 2016). Inhibition of mTORC1 leads to several downstream events including the inhibition of cellular proliferation, cap-dependent translation and stimulation of autophagy, a catabolic mechanism involving self-digestion of organelles and long-lived proteins (Sehgal, 2003; Dowling *et al.*, 2010). Because autophagy is considered a cell survival mechanism (Ohsumi, 2014; Martinet *et al.*, 2014a), a growing body of evidence links impaired autophagy to many pathophysiological processes, most of which are chronic and dependent on long-term treatment (Jiang and Mizushima, 2014). Therefore, major efforts are being made to stimulate autophagy in several disease models (Rubinshtein *et al.*, 2007; Liao *et al.*, 2012; Wirawan *et al.*, 2012; Nixon, 2013). Nutrient deprivation is a strong stimulus of the autophagic process, but because this approach is unsuitable for chronic treatment *in vivo*, pharmacological interventions remain the most feasible choice. Rapalogs are therefore frequently used for their ability to promote autophagy (Verhey *et al.*, 2007; Crazzolara *et al.*, 2009; Zhai *et al.*, 2014; Kim and Guan, 2015). Remarkably, even though several methods have been applied to administer everolimus chronically in mice, little or no information is available regarding autophagy induction after long-term mTORC1

inhibition *in vivo*. Osmotic minipumps represent an elegant method to administer the drug continuously in preclinical *in vivo* studies. The devices are easy to use, provide constant drug plasma levels for various periods depending on the chosen model and induce only minimal stress in the animals (Cameron *et al.*, 2014). Unlike other widely implemented techniques such as intraperitoneal injections or oral gavage, osmotic minipumps, once implanted, require little attention from the investigator. However, sometimes the manufacturer's recommendations on the compatibility of some solvents with the osmotic minipump are neglected and organic solvents are used in far higher concentrations than approved. This incorrect use may account for unreliable pumping rates and imprecise data interpretation. Consequently, the first objective of the present study was to develop a vehicle that ensures maximal everolimus stability at body temperature over long periods while not compromising the physical integrity of the osmotic minipump. The second aim was to investigate the effect of everolimus treatment following short-term (3-days) and continuous chronic administration (28 days) on mTORC1 signalling and autophagy in mice. To this end, we have chosen to analyse liver tissue of treated animals because this organ is widely known to react in a quick and robust manner to induction of autophagy (Mizushima *et al.*, 2004; Martinet *et al.*, 2013).

Methods

Everolimus solution

Everolimus (Novartis Institutes for Biomedical Research) was dissolved in a vehicle composed of 50% (v/v) DMSO (Acros Organics, 167850010), 40% (v/v) propylene glycol (Fagron, 610948) and 10% (v/v) absolute ethanol (Fisher Chemicals, E/0650DF/17). The mixture was supplemented with 0.4 $\mu\text{L}\cdot\text{mL}^{-1}$ Tween 20 (Sigma-Aldrich, 1379) to increase the solubility of everolimus and was stored light-protected at -20°C as a 10 or 20 mM stock solution for up to 1 month. For stability tests, the everolimus stock was

Figure 1

Everolimus is stable at 37°C for at least 28 days in vehicle solution. A 10 mM everolimus stock was made in the proposed vehicle (see text for details) and stored light-protected at 37°C. At different time points (0, 10 or 28 days), J774A.1 murine macrophage cells were treated with a serial dilution containing 0.01 nM to 1 μ M everolimus (three replicates per treatment). After 4 h of treatment, cells were lysed and subjected to Western blotting (detection of p70S6K phosphorylation at Thr³⁸⁹).

incubated at 37°C for different time intervals, then diluted in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum to treat murine J774A.1 macrophages. Four hours after treatment, the phosphorylation status of ribosomal protein S6 kinase B1 (p70S6K) was examined by Western blotting.

Mice

All animal care and experimental procedures were approved by the ethics committee of the University of Antwerp (no. 2012-54). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015). We used female GFP-microtubule-associated protein light chain 3 (LC3) transgenic mice (RIKEN BioResource Center, strain GFP-LC3 #53), containing a GFP fused to rat LC3 under control of the chicken β actin promoter. The mice were housed in a temperature-controlled environment (six animals per cage) with 12 h light/dark cycles and received food and water *ad libitum*. These mice are widely used in autophagy research (Mizushima *et al.*, 2004). The mice were selected randomly from the pool of all cages and divided into groups. All analyses were carried out without prior knowledge of the treatments. Mice received a numerical code throughout the whole experiment.

In vivo administration of everolimus by osmotic minipumps

Female GFP-LC3 mice (13–15 weeks old) were randomly divided into groups ($n = 6$ per group) and anaesthetised using sevoflurane. Hair was shaved off in the neck area, and a small incision was made between the ears. Using a haemostat, a

Figure 2

Short-term (3 days) everolimus treatment results in mTOR inhibition *in vivo*. Osmotic minipumps releasing either everolimus (EVR, 1.5 mg·kg⁻¹ per day) or vehicle were implanted in GFP-LC3 mice. After 3 days, mice were given an I.P. injection of 100 mg·kg⁻¹ chloroquine and were killed. Liver samples were collected and homogenized. Western blotting was performed to measure the phosphorylation status of Akt1 (Ser⁴⁷³), mTOR (Ser²⁴⁴⁸) and S6rp (Ser^{235/236}) as well as the expression of Cdk2. ACTB was used as a loading control. Representative blots are shown ($n = 6$ for both groups, * $P < 0.05$, significantly different from vehicle).

Figure 3

Short-term (3 days) everolimus treatment results in autophagy stimulation *in vivo*. Osmotic minipumps releasing either everolimus (EVR, $1.5 \text{ mg} \cdot \text{kg}^{-1}$ per day) or vehicle were implanted in GFP-LC3 mice. After 3 days, mice were given an I.P. injection of $100 \text{ mg} \cdot \text{kg}^{-1}$ chloroquine and were killed. Liver samples were collected. (A) Western blotting was performed to assess the ratio of free GFP/GFP-LC3, LC-II/LC3-I and accumulation of SQSTM1. (B) Liver samples were immunohistochemically stained for LC3 (upper panels) or analysed with fluorescence microscopy for GFP positive puncta (lower panels). Scale bar = $100 \mu\text{m}$. (C) Autophagic vacuoles (arrow heads) were detected in ultrathin sections using TEM. Scale bar = $1 \mu\text{m}$ ($n = 6$ for both groups, $*P < 0.05$, significantly different from vehicle).

subcutaneous pocket wide enough for an osmotic minipump was created. The pocket was flushed with saline and an osmotic minipump (Alzet) was inserted. After closing the incision with sterile sutures, a single dose of $7.5 \text{ mg} \cdot \text{kg}^{-1}$ ketorolac tromethamine (Ketalar(R), Pfizer, USA) was subcutaneously administered to reduce any postoperative pain. Two types of osmotic minipumps were used to deliver either vehicle or everolimus (Novartis, Switzerland) at $1.5 \text{ mg} \cdot \text{kg}^{-1}$ per day. Model 1003D was used to treat mice for 3 days, while model 1004 offered a long-term delivery of 28 days. Calculations of the required everolimus concentrations were made per individual mouse based on its weight and using the on-line tool provided by Alzet. At the end of the experiment, mice were injected intraperitoneally with $100 \text{ mg} \cdot \text{kg}^{-1}$ chloroquine (Sigma-Aldrich, 50–63-5) dissolved in saline. Three hours later, blood was taken via the retro-orbital plexus of anaesthetised mice (ketamine (Rompun(R), Beyer, Germany) $100 \text{ mg} \cdot \text{kg}^{-1}$ and xylazine $10 \text{ mg} \cdot \text{kg}^{-1}$, i.p.). The animals were subsequently killed using an overdose of sodium pentobarbital ($250 \text{ mg} \cdot \text{kg}^{-1}$, I.P.). Samples of the liver were collected for Western blotting, immunohistochemical analysis, fluorescence microscopy and transmission electron microscopy

(TEM). Administration of the vehicle alone had no effect on mTORC1 signalling or autophagy *in vivo* (not shown).

In vivo administration of everolimus by oral gavage

Female GFP-LC3 transgenic mice (RIKEN BioResource Center, strain GFP-LC3 #53) were divided into two groups ($n = 6$ per group, mice 13–15 weeks old) receiving either everolimus or vehicle [$25 \mu\text{L}$, diluted with $175 \mu\text{L}$ of Dulbecco's phosphate buffered saline (Gibco, 14190094)]. Oral gavage was performed using a 20G flexible catheter after removing the needle (Terumo; outer diameter 1.10 mm , inner diameter 0.80 mm) every other day for a period of 56 days. Following this period, mice were injected with $100 \text{ mg} \cdot \text{kg}^{-1}$ chloroquine I.P. and killed 3 h later.

Western blotting

Cells were lysed in an appropriate volume of Laemmli sample buffer (Bio-Rad, 1610737). Tissue samples were first homogenized in RIPA buffer (Sigma-Aldrich, R0278) containing protease and phosphatase inhibitors (Roche Diagnostics, 04693159001; 04906845001) using a Precellys 24

Figure 4

Continuous long-term (28 days) everolimus treatment induces drug tolerance *in vivo*. GFP-LC3 mice received osmotic minipumps releasing either everolimus (EVR, 1.5 mg·kg⁻¹ per day) or vehicle for a period of 28 days. Prior to killing, mice received an i.p. injection of 100 mg·kg⁻¹ chloroquine. Western blotting was performed to measure the phosphorylation status of Akt1 (Ser⁴⁷³), mTOR (Ser²⁴⁴⁸) and S6rp (Ser) as well as the expression of Cdk2. ACTB was used as a loading control. Representative blots are shown (*n* = 6 for both groups, **P* < 0.05, significantly different from vehicle).

homogenizer (Bertin technologies). Samples were then heat-denatured for 5 min in boiling water and loaded on pre-casted Novex Bolt 4–12% Bis-Tris gels (Invitrogen, BG-04120). After electrophoresis, proteins were transferred to an Immobilon-FL PVDF membrane (Millipore, IPFL00010) according to standard procedures. Membranes were blocked for 1 h in Odyssey blocking buffer (LI-COR Biosciences, 972-40100) diluted 1:5 with PBS. After blocking, membranes were probed overnight at 4°C with primary antibodies diluted in Odyssey blocking buffer, followed by 1 h incubation with IRDye-labelled secondary antibodies at room temperature. Antibody detection was achieved using an Odyssey SA infrared imaging system (LI-COR Biosciences). The intensity of the protein bands was quantified using Image Studio software.

The following mouse antibodies were used: anti-actin, β (ACTB) (clone AC-15, Sigma-Aldrich, A5441), anti-Cdk2 (BD Transduction Laboratories, C18520) and anti-LC3 (clone 5F10, Nanotools, 0231-100/LC3-5F10). Rabbit antibodies included anti-GFP (ab6556) from Abcam and anti-sequestosome 1 (SQSTM1) (#5114), anti-phospho-p70S6K(Thr³⁸⁹) (#9205), anti-p70S6K (#9202), anti-phospho-Akt(Ser⁴⁷³) (#9271), anti-Akt (#9272), anti-phospho-S6 ribosomal protein (S6rp) (Ser^{235/236}) (#2211), anti-S6rp (#2217), anti-phospho-unc-51 like autophagy activating kinase 1 (ULK1)(Ser⁷⁵⁷) (#6888), anti-ULK1 (#8054), anti-phospho-mTOR(Ser²⁴⁴⁸) (#2971) and anti-mTOR (#2972) from Cell Signalling. IRDye-labelled secondary antibodies (goat anti-mouse IgG, 926-68070 and goat anti-rabbit IgG, 926-32211) were purchased from LI-COR Biosciences.

Immunohistochemistry

Tissue samples were fixed in 4% neutral buffered formalin for 24 h prior to paraffin embedding. Thereafter, 5-μm-thick

sections were prepared and immunostained for LC3 using mouse anti-LC3 (clone 5F10, Nanotools, 0231-100/LC3-5F10) followed by anti-mouse Envision+ (Dako, K4001) as previously described (Martinet *et al.*, 2013; Martinet *et al.*, 2014b). Quantification was done by taking five images per section at random, and ImageJ software was used for analysis of the LC3-positive area.

Fluorescence microscopy

Formalin-fixed, paraffin-embedded tissue sections (5 μm thick) were deparaffinized with toluene followed by washing in isopropanol and water. Samples were finished with Vectashield mounting medium containing DAPI (Vector laboratories, H-1200). Images were taken using an EVOS FL Auto Cell Imaging System (ThermoFisher) and quantification of GFP puncta was done with ImageJ software.

Transmission electron microscopy

Tissues were prepared for TEM analysis as previously described (Martinet *et al.*, 2014a). A FEI Tecnai microscope was used to examine ultrathin sections at 80–120 kV. Quantification of autophagic vacuoles was achieved by manually counting vacuoles in five images per section taken at random.

Everolimus plasma concentration

A sample of 100 μL plasma was mixed with an equal volume of TitriPUR 0.1 M ZnSO₄ precipitation reagent (Merck Millipore, 1.08879.1000) and 250 μL [13C2D4]everolimus internal standard solution (5 μg·L⁻¹, Analytical Services International) dissolved in 100% methanol (LCMS reagent, J.T. Baker, 9830-02). Samples were vortexed for 1 min, kept 5 min in an ultrasonic bath and centrifuged for 10 min at 18 400× *g*. Twenty microlitre of the clear supernatant was injected in an online SPE trapping column (Luna 3 μm C18 10 × 4.6 mm, Phenomenex, 00D-4251-E0). By using a 60/40

Figure 5

Continuous long-term (28 days) everolimus treatment results in a decrease in autophagic markers *in vivo*. GFP-LC3 mice received osmotic minipumps releasing either everolimus (EVR, 1.5 mg kg⁻¹ day⁻¹) or vehicle for a period of 28 days. Prior to killing, mice received an i.p. injection of 100 mg kg⁻¹ chloroquine. (A) Western blotting was performed to assess the ratio of free GFP/GFP-LC3 and LC3-II/LC3-I as well as the accumulation of SQSTM1. (B) Liver samples were immunohistochemically stained for LC3 (upper panels) or analysed with fluorescence microscopy for GFP positive puncta (lower panels). Scale bar = 100 µm. (C) Autophagic vacuoles (arrow heads) were detected in ultrathin sections using TEM. Scale bar = 1 µm ($n = 6$ for both groups, * $P < 0.05$, significantly different from vehicle).

methanol/water mixture, the analyte was eluted onto an analytical column (Zorbax Eclipse XBD-C18 1.8 µm 4.6 × 50 mm, Agilent, 925975-902) in a high-speed online HPLC tandem MS system (Agilent HPLC 1260 connected to a 6490 Triple Quadrupole Mass Spectrometer). The mobile phase used for chromatography consisted of an isocratic mixture of water/methanol (5/95) in which 2 mM ammonium acetate and 0.5% formic acid were dissolved. The analytical column temperature was kept at 60°C.

For detection, an Agilent 6490 quadrupole mass spectrometer was used in positive mode. Nitrogen was employed as nebulizing and collision gas. The jetstream settings were as follows: drying gas temperature 200°C, drying gasflow 14 L·min⁻¹, sheath gas temperature 250°C with a flow of 11 L·min⁻¹, capillary voltage 4500 V.

Data and statistical analysis

The data and statistical analysis in this study comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). All data are presented as box-plots (showing medians and interquartile ranges with whiskers representing ranges) and were

analysed with the Mann–Whitney *U*-test using GraphPad Prism software (version 6.0). A probability value <0.05 was considered significant.

Results

Everolimus stability

Different solvent mixtures were prepared to investigate the stability of everolimus in a vehicle compatible with an osmotic minipump for long periods. As described previously (Simamora *et al.*, 2001), adding a benzoate buffer would increase solubility of everolimus. However, this acidic environment resulted in diminished activity of the drug after only 3 days of incubation at 37°C (data not shown). Addition of water to the mixture equally resulted in accelerated degradation of the drug. Based on the physico-chemical properties of everolimus, a mixture of 50% DMSO, 40% propylene glycol and 10% ethanol supplemented with 0.4 µL·mL⁻¹ Tween 20 was eventually used to prepare a 10 mM everolimus stock solution. The stock was incubated light-protected at 37°C, serially diluted

Figure 6

Intermittent long-term (56 days) everolimus treatment rescues mTORC1 sensitivity to therapy *in vivo*. GFP-LC3 mice were administered 3 mg·kg⁻¹ everolimus (EVR) by oral gavage every other day for a period of 56 days. At the end of the experiment, mice were then given an I.P. injection containing 100 mg·kg⁻¹ chloroquine and were killed 3 h later. Western blotting was performed to measure the phosphorylation status of Akt1 (Ser⁴⁷³), mTOR (Ser²⁴⁴⁸) and S6rp (Ser^{235/236}) as well as the expression of Cdk2. ACTB was used as a loading control. Representative blots are shown ($n = 6$ for both groups, * $P < 0.05$, significantly different from vehicle).

in RPMI medium and used to treat J774A.1 murine cells at different time points (0, 10 and 28 days). The stability of everolimus was assessed by its ability to inhibit the phosphorylation of p70S6K at Thr³⁸⁹, a highly sensitive target of mTORC1. From day 0 until day 10, the phosphorylation of p70S6K was fully suppressed by subnanomolar concentrations of everolimus (Figure 1), whereas after 28 days, only concentrations above 1 nM led to complete dephosphorylation of p70S6K. Everolimus did not influence the expression of total p70S6K.

Short-term (3 days) everolimus treatment

An osmotic minipump filled with everolimus solution was inserted subcutaneously in GFP-LC3 mice. Mice were intraperitoneally injected with 100 mg·kg⁻¹ chloroquine 3 h prior to kill to block autophagic flux. The minipump delivered the drug for 3 days at a constant rate of 1.5 mg·kg⁻¹ per day, yielding a plasma concentration of 630 ± 34 nM at the time of killing. Western blot analysis of liver tissue from everolimus-treated GFP-LC3 mice revealed no effect of short-term everolimus administration on Akt1 phosphorylation upstream of mTOR while the phosphorylation of both mTOR and its downstream target S6rp was significantly suppressed compared to the vehicle-treated control animals (Figure 2). This was accompanied with a decrease of the mTORC1-controlled cell cycle progression regulator cyclin-dependent kinase 2 (Cdk2) expression, a crucial protein involved in cell proliferation (Woods, 2010), when normalized with the ACTB loading control.

To evaluate the induction of autophagy *in vivo*, Western blotting was used to assess several autophagy markers such as the accumulation of SQSTM1, the ratio free GFP to GFP-LC3 and the ratio endogenous LC3-II to LC3-I. Ratios of free

GFP/GFP-LC3 and LC3-II/LC3-I showed a significant increase following everolimus treatment, indicating effective autophagy induction (Figure 3A). Increased levels of SQSTM1 were also observed. Immunohistochemical analysis of LC3 in formalin-fixed liver tissue showed a significant increase in LC3 dots in everolimus-treated animals (Figure 3B, upper panel). Enhanced GFP fluorescence in the everolimus group further indicated autophagy induction (Figure 3B, lower panel). Lastly, TEM analysis confirmed the formation of numerous autophagic vacuoles after administration of everolimus (Figure 3C). It should be noted that the results described above were obtained after injecting mice with chloroquine to prevent protein degradation in autolysosomes and to improve the accumulation of autophagy markers. Results of autophagy induction without chloroquine injection are shown in Figure S1. In the absence of chloroquine, levels of SQSTM1 were decreased in the treated animals while ratios of LC3-II/LC3-I and free GFP/GFP-LC3 were increased implying functional autophagic flux.

Long-term (28 days) continuous everolimus treatment

To administer everolimus chronically, osmotic minipumps were implanted in GFP-LC3 mice to deliver the drug for 28 days at the same constant rate of 1.5 mg·kg⁻¹ per day. This approach yielded a plasma concentration of 503 ± 58 nM at day 28. This concentration, while lower than the one found after short-term treatment, most likely due to everolimus degradation, was still far above the 5 nM threshold for *in vivo* mTORC1 inhibition (Tanaka *et al.*, 2008; Fox *et al.*, 2010; O'Reilly and McSheehy, 2010). Nonetheless, everolimus was unable to dephosphorylate mTOR or S6rp. Akt1 – upstream of mTOR – showed

Figure 7

Intermittent long-term (56 days) everolimus treatment fails to induce autophagy *in vivo*. GFP-LC3 mice were administered 3 mg kg⁻¹ everolimus (EVR) by oral gavage every other day for a period of 56 days. At the end of the experiment, mice were then given an I.P. injection containing 100-mg·kg⁻¹ chloroquine and were killed 3 h later. (A) Western blotting was performed to assess the ratio of free GFP/GFP-LC3 and LC3-II/LC3-I as well as the accumulation of SQSTM1. (B) Liver samples were immunohistochemically stained for LC3 (upper panels) or analysed with fluorescence microscopy for GFP positive puncta (lower panels). Scale bar = 100 µm. (C) Autophagic vacuoles (arrow heads) were detected in ultrathin sections using TEM. Scale bar = 1 µm (*n* = 6 for both groups, **P* < 0.05, significantly different from vehicle).

significant hyperphosphorylation while the downstream target Cdk2 was overexpressed in the everolimus-treated animals (Figure 4). Western blot results also revealed lower expression levels of SQSTM1 accompanied with a decrease in the ratio of both free GFP/GFP-LC3 and LC3-II/LC3-I in the everolimus-treated group versus controls (Figure 5A), suggesting a decline (rather than an increase) in autophagy. Impaired autophagy was confirmed by immunohistochemistry (lack of LC3 puncta, Figure 5B, upper panel), fluorescence microscopy (decrease in GFP fluorescence, Figure 5B, lower panel) and TEM (decreased number of autophagic vesicles, Figure 5C). Results without chloroquine injection prior to kill also showed a decline in autophagy in everolimus-treated animals as demonstrated by decreased LC3-II/LC3-I and free GFP/GFP-LC3 ratios as well as a reduction of GFP positive puncta (Figure S2).

Long-term (56 days) intermittent everolimus treatment

In an attempt to bypass everolimus resistance after continuous everolimus treatment, we decided to perform another set of experiments in which everolimus is administered to

mice intermittently. This approach allows a drug-free period during which liver cells may reactivate mTORC1. The drug, or vehicle, was administered to female GFP-LC3 mice by oral gavage every other day for a period of 56 days, thereby effectively treating mice for a total of 28 days. Mice were given a dose of 3 mg·kg⁻¹, which resulted in a plasma concentration of 603 ± 112 nM in blood withdrawn 2 h after oral gavage. Treated mice revealed a marked sensitivity to the drug as indicated by the strong dephosphorylation of S6rp. However, Akt1 phosphorylation was slightly increased while mTOR phosphorylation and expression of Cdk2 were unaffected (Figure 6). Autophagy induction was not observed (Figure 7A–C).

Because failure to induce autophagy was not due to depletion of autophagy specific proteins such as SQSTM1 or LC3, we investigated the activation status of the critical autophagy initiator ULK1 (Lee and Tournier, 2011; Chan, 2012). Under normal circumstances, mTORC1 inactivates ULK1 by phosphorylating the protein at serine 757, which leads to inhibition of autophagy. Inhibition of mTORC1 dephosphorylates ULK1 and triggers initiation of autophagy (Ganley *et al.*, 2009; Nakagawa *et al.*, 2012). We observed a decrease in

Figure 8

Frequency of everolimus administration affects ULK1 phosphorylation *in vivo*. Osmotic minipumps releasing either everolimus ($1.5 \text{ mg} \cdot \text{kg}^{-1}$ per day) or vehicle were implanted in GFP-LC3 mice for 3 days (short-term) or 28 days (continuous long-term). Alternatively, GFP-LC3 mice were administered $3 \text{ mg} \cdot \text{kg}^{-1}$ everolimus by oral gavage every other day for a period of 56 days (intermittent). mTORC1-specific phosphorylation of ULK1 at Ser⁷⁵⁷ was measured in the liver by Western blotting. The ratio phosphorylated of protein/total protein was used to assess the degree of phosphorylation. Representative blots are shown ($n = 6$ for all groups, $*P < 0.05$, significantly different from vehicle).

ULK1 phosphorylation following short-term everolimus treatment (Figure 8). However, phosphorylation increased significantly when the drug was given continuously for a long period (Figure 8). Intermittent administration failed to have any effect (Figure 8).

Discussion and conclusions

Since being FDA approved, everolimus has been used in immunosuppressive therapy and has recently also been evaluated clinically for the treatment of numerous disorders, in particular cancer (Verheye *et al.*, 2007; Mueller *et al.*, 2008; Wander *et al.*, 2011; Martinet *et al.*, 2014a). One of the crucial *in vivo* effects of everolimus pertains to its ability to stimulate autophagy. However, despite the chronic nature of most disorders in which the drug has been tested, little is known about its efficacy in inducing autophagy following long-term treatment. This study was initially designed to compare the *in vivo* effects of a short- and long-term treatment with everolimus on mTORC1 inhibition and autophagy induction using osmotic minipumps implanted subcutaneously in GFP-LC3 transgenic mice. Given the chronic nature of the

study, we first tested several solvent mixtures to ensure optimal solubility and stability of everolimus at physiological temperatures over long time spans. A vehicle composed of 50% DMSO, 40% propylene glycol and 10% ethanol supplemented with $0.4 \mu\text{L} \cdot \text{mL}^{-1}$ Tween 20 was found to solubilize everolimus and to keep it stable at 37°C for at least 28 days. Because organic solvents at high concentrations can damage the inner compartments of an osmotic minipump, the concentration of each component in the solvent mixture was carefully chosen to avoid disturbance of the pumping rate and the generation of unreliable data. Even though some activity was lost during the *in vitro* incubation of everolimus in the above-mentioned solvent mixture at 37°C , everolimus retained most of its activity, resulting in full mTORC1 inhibition. To our knowledge, no other vehicles have been described in which everolimus remains stable at this temperature for long periods.

Short-term (3 days) treatment of GFP-LC3 mice with everolimus led to strong mTORC1 inhibition in the liver as demonstrated by a significant reduction of S6rp and mTOR phosphorylation. Moreover, autophagy was clearly stimulated as shown by Western blotting, immunohistochemistry, fluorescence microscopy and TEM. In contrast, continuous

Figure 9

Summary scheme of the main results. Short-term everolimus administration inhibits phosphorylation of the mTORC1 substrates S6rp and ULK1 resulting in robust induction of autophagy. Continuous long-term administration of everolimus triggers adaptation mechanisms resulting in hyperphosphorylation of ULK1 and inhibition of autophagy. Long-term administration of everolimus intermittently rescues responsiveness to the drug only in the most sensitive substrate (S6rp) without any effect on autophagy.

long-term (28 days) administration of everolimus resulted in diminished autophagy as compared with the non-treated control group. This finding could be explained by a complete failure of everolimus to inhibit mTORC1 signalling in the liver tissue of these mice, as previously reported following chronic exposure to rapamycin (Drake *et al.*, 2013). Of note, the plasma levels of everolimus following continuous administration were lower than those measured during short-term treatment (503 ± 58 nM vs. 630 ± 34 nM respectively). This reduction is most likely due to a certain degree of everolimus degradation in the osmotic minipump, or else the result of individual variability in the pharmacokinetics of the drug as shown in humans (Budde *et al.*, 2004). Nonetheless, because everolimus is known to inhibit mTORC1 *in vivo* at plasma concentrations as low as 5 nM (Tanaka *et al.*, 2008; Fox *et al.*, 2010; O'Reilly and McSheehy, 2010), this reduction could not explain the loss of everolimus efficacy.

A plausible alternative explanation might relate to the resistance to everolimus following long-term treatment, which is a clinically relevant and well-described phenomenon in renal cell carcinoma (RCC), prostate cancer and some other tumour types (Tsaur *et al.*, 2011; Hassan *et al.*, 2014; Juengel *et al.*, 2014; Wagle *et al.*, 2014). These tumours, while initially reacting well to everolimus therapy, often progress to a resistant form in the long run. This resistance is only seen following allosteric mTORC1 inhibition leaving tumours still responsive to a novel class of mTOR kinase inhibitors that are currently being tested in

clinical trials (Guichard *et al.*, 2015). A number of mechanisms including absence of the mTORC1-S6rp negative feedback loop, mutations of the allosteric binding site of mTOR or hyperactivation of the Akt1-mTORC1 pathway have been proposed as possible explanations for the acquired resistance to allosteric mTORC1 inhibitors (Carew *et al.*, 2011; Porta *et al.*, 2014; Saran *et al.*, 2015; Yang *et al.*, 2015). For example, long-term everolimus treatment of RCC cells *in vitro* resulted in everolimus resistance, a condition that was characterized by a high phosphorylation status of both Akt1 and S6rp (Juengel *et al.*, 2012; Hassan *et al.*, 2014; Juengel *et al.*, 2014), which are up and downstream of mTORC1 respectively. Moreover, a significant up-regulation of the cell cycle regulator Cdk2 was observed in RCC cells after prolonged everolimus treatment. Interestingly, we also found these features in the liver samples of GFP-LC3 mice after continuous, long-term everolimus treatment. It is therefore tempting to speculate that analogous to a tumour exposed to long-term mTORC1 inhibition, the high synthesis rate of liver cells (Turley *et al.*, 1981; Shahbazian *et al.*, 1987) drives the initiation of adaptation mechanisms, resulting in hyperactivation of the Akt1-mTORC1 pathway and drug tolerance. Therefore, we hypothesized that a drug-free period could relieve pressure on the cells and prevent mTORC1 resistance to everolimus. Indeed, the use of intermittent administration regimens in avoiding resistance against everolimus is supported by a recent case of a patient with malignant insulinoma (Baratelli *et al.*, 2014) and has also been shown to minimize undesirable immunosuppressive effects of everolimus without affecting its activity on tumours (Boulay *et al.*, 2004).

Because peritonitis is a common side effect of intraperitoneal injections with solutions containing DMSO, we delivered everolimus by oral gavage. We decided to use an intermittent regimen delivering everolimus every other day because of the very short half-life (6 h) in mice (O'Reilly and McSheehy, 2010). The experiment lasted 56 days during which GFP-LC3 mice effectively received everolimus (or vehicle) for 28 days to allow comparison with the group receiving everolimus continuously. The delivered dose ($3 \text{ mg} \cdot \text{kg}^{-1}$) yielded plasma concentrations comparable with those obtained when using osmotic minipumps. Strikingly, intermittent treatment resulted in a strong reduction in S6rp phosphorylation in the liver. Compared with continuous administration, a drug-free period effectively attenuated the development of tolerance and successfully extended the period during which the liver remained (partially) sensitive to treatment with everolimus. These results may add new insights to the field of oncology as efficacy of everolimus in several tumours has been correlated with its ability to inhibit S6rp phosphorylation (O'Reilly and McSheehy, 2010; Li *et al.*, 2014; Masuda *et al.*, 2014). However, there was no sign of autophagy induction in the intermittently treated group despite the apparent mTORC1 sensitivity to everolimus. Because mTORC1 substrates are not equally sensitive to allosteric mTORC1 inhibition (Thoreen and Sabatini, 2009; Thoreen *et al.*, 2009; Benjamin *et al.*, 2011; Yoon and Roux, 2013; Saran *et al.*, 2015), we hypothesized that less sensitive substrates may become uncoupled from this inhibition more rapidly resulting in partial mTORC1 inhibition. For instance, while phosphorylation of the very sensitive substrate S6rp

may still be suppressed, the less sensitive ULK1 can explain why autophagy was not induced. ULK1 is the main effector through which both mTORC1 and the AMP-activated protein kinase are able to initiate autophagy (Kim *et al.*, 2011; Nakagawa *et al.*, 2012; Russell *et al.*, 2013; Saran *et al.*, 2015). Consequently, we investigated the phosphorylation status of ULK1 at the mTORC1 specific Ser⁷⁵⁷ site. In accordance with its ability to induce autophagy, short-term administration of everolimus promptly inhibited ULK1 phosphorylation. This effect was lost following long-term treatment, either continuously or intermittently, resulting in significant up-regulation of ULK1 phosphorylation and inhibition of autophagy.

Taken together, our data indicate that allosteric short-term (3 days) mTORC1 inhibition by everolimus in GFP-LC3 mice triggers dephosphorylation of the mTORC1 target S6rp and induction of autophagy (Figure 9). However, if mice were continuously exposed to everolimus for longer periods (28 days), mTORC1 resistance and autophagy inhibition occurred (Figure 9). Long-term (56 days) intermittent administration of everolimus led to dephosphorylation of S6rp, but did not rescue the autophagy-inducing properties of everolimus (Figure 9). These findings are particularly interesting in cancer research since resistance to everolimus is very common in tumours and S6rp dephosphorylation mediated by the drug has been linked to its anti-tumour activity.

Acknowledgements

This work was supported by the Fund for Scientific Research (FWO)-Flanders (project G.0160.13 N) and the University of Antwerp (BOF). The FEI Tecnai transmission electron microscope was purchased with support of the Hercules Foundation. The authors thank Dr. Noboru Mizushima (Tokyo Medical and Dental University, Japan) for providing GFP-LC3 mice and Rita Van Den Bossche, Hermine Fret, Anne-Elise Van Hoydonck, Lieve Svensson and Francis Terloo for their excellent technical support.

Author contributions

D.M.G.R.Y., M.W., N.H. and K.A. study conception and design; K.A.: All the Western Blotting data, histology data and surgical procedures. D.D.M. and A.S.: All the plasma concentration measurements. L.A. performed the oral gavages. T.J.-P.: All the electron microscopy data, acquisition of data; K.A.: Analysis of the Western Blotting, histology and electron microscopy data. D.D.M. and A.S.: Analysis of the plasma concentration data. M.W. and D.M.G.R.Y.: Interpretation of the data in general. L.K.: Statistical analysis, analysis and interpretation; All authors helped drafting the manuscript. K.A.: Introduction, Methods and Results. L.A., D.D.M., T.J.-P. and A.S.: Results and critical revision. L.K.: Discussion (clinical aspects) and critical revision. N.H.: Discussion/Results (Plasma concentration and clinical implications). M.W. and D.M.G.R.Y.: Discussion and critical revision, drafting of manuscript; All authors approved the final version to be submitted and agreed to be accountable.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This [Declaration](#) acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

<http://dx.doi.org/10.1111/bph.13626>

Figure S1 Short-term (3 days) everolimus treatment without chloroquine. Osmotic minipumps releasing either everolimus (EVR, 1.5 mg kg⁻¹ day⁻¹) or vehicle were implanted in GFP-LC3 mice. Animals were killed after 3 days. Liver samples were collected. (A) Western blotting was performed to assess the ratio of free GFP/GFP-LC3, LC-II/LC3-I and accumulation of SQSTM1. (B) Liver samples were immunohistochemically stained for LC3 (upper panels) or analyzed with fluorescence microscopy for GFP positive puncta (lower panels). Scale bar = 100 µm. (C) Autophagic vacuoles (arrow heads) were detected in ultrathin sections using transmission electron microscopy. Scale bar = 1 µm (*n* = 6 for both groups, **P* < 0.05 versus vehicle).

Figure S2 Continuous long-term (28 days) everolimus treatment without chloroquine. GFP-LC3 mice received osmotic minipumps releasing either everolimus (EVR, 1.5 mg kg⁻¹ day⁻¹) or vehicle for a period of 28 days. (A) Western blotting was performed to assess the ratio of free GFP/GFP-LC3 and LC3-II/LC3-I as well as the accumulation of SQSTM1. (B) Liver samples were immunohistochemically stained for LC3 (upper panels) or analyzed with fluorescence microscopy for GFP positive puncta (lower panels). Scale bar = 100 µm. (C) Autophagic vacuoles (arrow heads) were detected in ultrathin sections using transmission electron microscopy. Scale bar = 1 µm (*n* = 6 for both groups, **P* < 0.05 versus vehicle). Supporting info item.