Journal of Hepato-Biliary-Pancreatic Sciences

Impact of very early introduction of everolimus on liver regeneration after partial liver transplantation in rats

Journal:	*Journal of Hepato-Biliary-Pancreatic Sciences*
Manuscript ID	JHBPS-2022-0502.R2
Wiley - Manuscript type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Hirata, Masaaki; Kyoto University, Department of Surgery Yagi, Shintaro; Kanazawa University, Department of Hepato-Biliary-Pancreatic Surgery and Transplantation Ito, Takashi; Kyoto University, Department of Surgery Masano, Yuki; Kyoto University, Department of Surgery Miyachi, Yosuke ; Kyoto University, Department of Surgery Yao, Siyuan; Kyoto University, Department of Surgery Sonoda, Mari; Kyoto University, Department of Surgery Masuda, Satohiro; Himeji Dokkyo University, Department of Clinical Pharmacy Haga, Hironori; Kyoto University Hospital, Department of Diagnostic Pathology Hatano, Etsuro; Kyoto University, Department of Surgery
ORGAN:	LIVER
RESEARCH FIELD:	TRANSPLANTATION
Keywords: please type five keywords that are identical to those described in the manuscript:	mTOR inihibitor, immunosuppressive regimen, partial liver graft, post-operative complications, rejection
Abstract:	Background/Purpose: This experimental study in rats aimed to investigate the impact of very early introduction (within 3 hours) of everolimus (EVR) + reduced-tacrolimus (TAC) after partial liver transplantation (LT) on liver regeneration, rejection, and survival. Methods: Based on appropriate dose of EVR + reduced-TAC in 70% hepatectomy (Experiment 1), allogeneic 30% partial LT (Experiment 2) and whole LT (Experiment 3) were performed. Results: After partial LT in EVR + reduced-TAC therapy, restoration of liver graft weight (to that of the whole liver) was delayed compared with standard dose TAC monotherapy (standard-TAC) on Day 3 (59.3% vs. 72.9%; P<0.001) and 14 (88.1% vs. 95.5%; P=0.01). And survival was 75%, which was not as high as the value of 100% observed for standard-TAC,

	because neither infection nor rejection could be prevented. By contrast, survival after whole LT was 100% as neither infection nor rejection occurred. Conclusions: The very early introduction of EVR + reduced-TAC after partial LT delayed liver regeneration, and made it difficult to manage the dose required to suppress both infection and rejection. On the other hand, EVR + reduced-TAC could be introduced safely very early after whole LT.

ORIGINAL ARTICLE

Title:

Impact of very early introduction of everolimus on liver regeneration after partial liver transplantation in rats

Authors:

Masaaki Hirata,[1] Shintaro Yagi,[2] Takashi Ito,[1] Yuki Masano,[1] Yosuke Miyachi,[1] Siyuan Yao,[1] Mari Sonoda,[1] Satohiro Masuda,[3] Hironori Haga,[4] and Etsuro Hatano[1]

Affiliations:

1. Department of Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan

2. Department of Hepato-Biliary-Pancreatic Surgery and Transplantation, Kanazawa University, Ishikawa, Japan

3. Department of Clinical Pharmacy, Faculty of Pharmaceutical Sciences, Himeji Dokkyo University, Hyogo, Japan

4. Department of Diagnostic Pathology, Kyoto University Hospital, Kyoto, Japan

Correspondence and Reprint Request to

Shintaro Yagi, M.D., Ph.D.

E-mail: yagi@med.kanazawa-u.ac.jp

Tel: +81-76-265-2369, Fax number: +81-76-234-4260

Word Count

3798

Table Count

2

Figure Count

5

Keywords

mTOR inhibitor; immunosuppressive regimen; partial liver graft; post-operative complications; rejection

ABSTRACT

Background/Purpose:

This experimental study in rats aimed to investigate the impact of very early introduction (within 3 hours) of everolimus (EVR) + reduced-tacrolimus (TAC) after partial liver transplantation (LT) on liver regeneration, rejection, and survival.

Methods:

Based on appropriate dose of EVR + reduced-TAC in 70% hepatectomy (Experiment 1), allogeneic 30% partial LT (Experiment 2) and whole LT (Experiment 3) were performed.

Results:

After partial LT in EVR + reduced-TAC therapy, restoration of liver graft weight (to that of the whole liver) was delayed compared with standard dose TAC monotherapy (standard-TAC) on Day 3 (59.3% *vs.* 72.9%; P<0.001) and 14 (88.1% *vs.* 95.5%; P=0.01). And survival was 75%, which was not as high as the value of 100% observed for standard-TAC, because neither infection nor rejection could be prevented. By contrast, survival after whole LT was 100% as neither infection nor rejection occurred.

Conclusions:

The very early introduction of EVR + reduced-TAC after partial LT delayed liver regeneration, and made it difficult to manage the dose required to suppress both infection and rejection. On the other hand, EVR + reduced-TAC could be introduced safely very early after whole LT.

ABBREVIATIONS

ALT, alanine aminotransferase

AST, aspartate aminotransferase

BUN, blood urea nitrogen

Cre, creatinine

DA, Dark Agouti

EVR, everolimus

LDLT, living-donor liver transplantation

LEW, Lewis

LT, liver transplantation

POD, post-operative day

RAI, rejection activity index

TAC, tacrolimus

T-Bil, total bilirubin

TCMR, T cell-mediated rejection

1. INTRODUCTION

The introduction of everolimus (EVR) allows less use of calcineurin inhibitors, which has led to greater preservation of renal function and reduced the risk of post-liver transplantation (LT) recurrence of hepatocellular carcinoma.[1-4] It has been suggested that early introduction of EVR after LT is more effective than late introduction with respect to yielding these benefits.[5-7] It may also be beneficial in preventing the development of donor-specific antibodies after LT.[8] Some studies report increased safety and renal protective effects after early introduction of EVR within 30 days of deceased-donor LT, although there were concerns about adverse effects of EVR, such as delayed wound healing and hepatic artery thrombosis.[6,9-13] In contrast to the response to sirolimus, another mammalian target of rapamycin inhibitor, there have been no reports of increased hepatic artery thrombosis in response to early introduction of EVR.[14] However, it remains unclear whether early introduction of EVR within 30 days after partial LT, as occurs with living-donor LT (LDLT), is safe, since EVR may inhibit liver regeneration due to its antiproliferative effects.[15,16]

Therefore, we performed 70% hepatectomy in a rat model to determine the doses of EVR and tacrolimus (TAC) for LT experiments, and to investigate the impact of EVR on liver regeneration. Subsequently, rat allogeneic 30% partial LT and whole LT were performed

to evaluate the impact of very early introduction of EVR, with or without reduced-TAC, on liver regeneration, T cell-mediated rejection (TCMR), and survival.

2. MATERIAL AND METHODS

2.1 Animals

Male Lewis (LEW) ($RT1^{l}$) rats (weighing 250–320 g, Japan SLC, Inc) and male Dark Agouti (DA) ($RT1^{av1}$) rats (weighing 230–290 g, Japan SLC, Inc) were used. Animals were housed under specific pathogen-free conditions in a temperature- and humidity-controlled environment with a 12 hour light/dark cycle. Rats were fed a standard diet (F-2; Oriental Bio Service) and tap water ad libitum. The experimental protocol was approved by the institutional ethics committee of Kyoto University (Medkyo-19610). All rats received humane care according to the Guide for the Care and Use of Laboratory Animals.

2.2 70% hepatectomy

The median and left liver lobes of male LEW rats were resected, as previously described.[17]

2.3 Allogeneic 30% partial LT and whole LT

Fully major histocompatibility complex-disparate livers from DA donors used as high-responder allogeneic models were transplanted orthotopically into LEW recipients (**Figure 1A**). Allogeneic 30 % partial LT and whole LT with hepatic artery reconstruction were performed as previously reported.[18-21] The 30 % partial graft (right and caudate lobes) was chosen to evaluate liver regeneration.[20] The detailed surgical procedures are described in the Supplemental Methods.

2.4 Immunosuppressive drugs

EVR (Certican; Novartis Pharma AG, Basel, Switzerland) and/or TAC (Prograf; Astellas Pharma, Inc., Ibaraki, Japan) were used as immunosuppressive drugs. Both drugs were diluted in 1 mL of distilled water and administered by oral gavage immediately after surgery (i.e., within 3 hours after hepatectomy or LT), and once daily every morning thereafter until sacrifice (**Figure 1B**). Whole blood trough concentrations were used for therapeutic drug monitoring of EVR and TAC. The target ranges for trough concentrations recommended for clinical practice were used. For EVR + reduced-TAC therapy, the trough concentrations are usually targeted at 10–15 ng/mL of the total sum of EVR (3–12 ng/mL) and TAC (3–5 ng/mL).[11,12] For EVR monotherapy, the EVR trough

concentrations are usually targeted at 5–10 ng/mL; for TAC monotherapy, the TAC trough concentrations are targeted at 5–15 ng/mL.[1,2,22] Distilled water (1 mL) was used as a non-immunosuppression control.

2.5 Experiment 1: The 70% hepatectomy model

Here, 70% hepatectomy was performed to determine the doses of EVR and TAC for LT experiments. First, two different doses of EVR monotherapy (1.3 mg/kg (EVR1.3) and 2.5 mg/kg (EVR2.5)) and one dose of TAC monotherapy (6.5 mg/kg (TAC6.5)) were tested, with reference to a previous report for healthy rats.[23] The doses were evaluated based on trough concentrations and renal function. Subsequently, the doses of EVR + reduced-TAC therapy were explored based on the doses used for each monotherapy. Finally, the impact of very early introduction (within 3 hours after hepatectomy) of EVR, with or without reduced-TAC, on liver regeneration was evaluated. Rats were sacrificed on post-operative day (POD) 1, POD3, POD7, and POD14 (n=4 for each).

2.6 Experiment 2: The 30% partial LT model

Here, 30% partial LT was performed to evaluate the impact of very early introduction (within 3 hours after LT) of EVR, with or without reduced-TAC, on liver regeneration,

TCMR, and survival. The doses used in Experiment 1 were selected because the liver volumes were similar. We evaluated whether the doses were appropriate in terms of suppression of both infection and TCMR, and their effects on renal function (referring to the trough concentrations). A 14-day survival study was performed for all groups. Rats that survived to POD14 were sacrificed to evaluate liver regeneration, liver histology, blood biochemistry, wound complications, patency of the reconstructed hepatic artery, and trough concentrations. Autopsies were performed to investigate the cause of death of rats that died before POD14. Patency of the hepatic artery was confirmed by bleeding after transection of the artery. Rats were also sacrificed on POD3 and POD5 to evaluate liver regeneration, liver histology, and trough concentrations during the early post-LT period.

2.7 Experiment 3: The whole LT model

Whole LT was performed to evaluate the impact of very early introduction (within 3 hours after LT) of EVR with reduced-TAC on 14-day survival, liver histology, patency of the reconstructed hepatic artery, wound complications, and trough concentrations. The doses were as in Experiment 2. A 14-day survival study was performed, and rats that survived to POD14 were sacrificed.

2.8 Trough concentrations of EVR and TAC, and blood biochemistry

Blood samples were collected on the morning of sacrifice. Trough concentrations of EVR and TAC in whole blood were measured in an electrochemiluminescence immunoassay using the Cobas 6000 and e601 systems (Roche Diagnostics), respectively. Liver function was assessed by measuring serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (T-Bil) levels in a Hitachi 7700 (Hitachi High-Tech Co). Renal function was assessed by measuring blood urea nitrogen (BUN) and creatinine (Cre) levels using Hitachi 7700 and LABOSPECT 008 systems (Hitachi High-Tech Co), respectively.

2.9 Histology and the Ki-67 labeling index

Liver tissues were collected at sacrifice and autopsy, fixed in buffered formalin, embedded in paraffin, cut and stained with hematoxylin-eosin and Giemsa stains, and used for microscopic observations. Tissue sections were evaluated by three independent investigators, including an expert LT pathologist (H.H). TCMR was scored using the rejection activity index (RAI) in accordance with the Banff criteria of hepatic allograft pathology.[24]

Hepatocyte proliferation was evaluated by staining deparaffinized hepatic sections for Ki-67 using a rabbit polyclonal anti-Ki-67 antibody (Abcam, ab15580). Ten high-power fields (×200) were randomly selected, and the Ki-67 labeling index (%) was calculated from the percentage of positive cells.

2.10 Statistical analysis

Continuous variables are presented as the mean ± standard deviation. For continuous variables, after confirming whether the data were normally distributed using the Shapiro-Wilk test, comparisons between two groups were made using an unpaired *t*-test or Mann-Whitney U test, and comparisons between multiple groups were made with the Tukey–Kramer test or Dunn test. For the survival study, a log-rank test was used. P values < 0.05 were considered statistically significant. All statistical analyses were performed using Prism 9 software (GraphPad Software Inc).

3. RESULTS

3.1 Experiment 1-1: Determination of the doses of EVR and TAC for LT experiments using the 70% hepatectomy model

The trough concentrations in the EVR1.3 group were within the target range for EVR +

reduced-TAC therapy, and those in the EVR2.5 and the TAC6.5 groups were within the target range for EVR or TAC monotherapy **(Table 1)**. Rats in the EVR2.5 group exhibited better renal function than the TAC6.5 group on POD14 **(Supplemental Figure 1)**. Therefore, as the appropriate dose for 70% hepatectomy, we chose 1.3 mg/kg of EVR for EVR + reduced-TAC therapy, and 2.5 mg/kg of EVR or 6.5 mg/kg of TAC for standard dose monotherapies. Trough concentrations in the EVR 1.3 mg/kg + TAC 6.5 mg/kg (EVR1.3 + TAC6.5) group were higher than the targeted values, suggesting a pharmacological interaction. Therefore, the doses of combined TAC were reduced to 3.0 mg/kg. In the EVR 1.3 mg/kg + TAC 3.0 mg/kg (EVR1.3 + TAC3.0) group, the trough concentrations were then within the target range, but renal protective effects were not achieved. An EVR 0.8 mg/kg + TAC 1.5 mg/kg (EVR0.8 + TAC1.5) group, in which EVR and TAC were reduced further in Experiment 2, was also tested. In the EVR0.8 + TAC1.5 group, renal protective effects were achieved, although the trough concentrations were slightly lower than the target values.

Finally, the impact on liver regeneration was evaluated in the following five dose groups (n=16 for each): Group 1) Control; Group 2) TAC6.5; Group 3) EVR2.5; Group 4) EVR1.3 + TAC3.0; and Group 5) EVR0.8 + TAC1.5. All rats showed normal liver histology without infection.

3.2 Experiment 1-2: Liver regeneration after 70% hepatectomy

Liver regeneration rates were defined according to the following formula: (remnant liver weight/body weight on sacrifice)/ (estimated whole liver weight (= resected liver weight/0.7)/pre-hepatectomy body weight) × 100 (%).[25]

Liver regeneration rates on POD3 in the EVR2.5 and the EVR1.5 + TAC0.8 groups were lower than those in the control and TAC6.5 groups, but became similar thereafter (**Figure 2A**). The Ki-67 labeling indices on POD1 in all groups that received EVR were lower than those in the control and TAC6.5 groups **(Figure 2B)**.

3.3 Experiment 2-1: 14-day survival study and histological findings in liver grafts after 30% partial LT

The 14-day survival rates in the EVR1.3 + TAC3.0 were 20%, while all rats in the TAC6.5 and the EVR2.5 groups survived (n=5 for each; **Figure 3**). In the EVR1.3 + TAC3.0 group, all rats that died before POD14 showed abscess formation or sinusoidal bacterial colonies in the autopsied liver grafts. A representative example is shown in **Figure 4A**. The bacterial colonies were also confirmed by Giemsa staining. Of the total 15 rats including those that were sacrificed on POD3 and POD5 (n=5 for each) in the

EVR1.3 + TAC3.0 group, eight (53%) exhibited abscess formation or sinusoidal bacterial colonies in the liver grafts. TCMR was not observed in all rats **(Table 2)**. The trough concentrations were higher than targeted on POD3, and they increased even more on POD5, suggesting immune over-suppression. Therefore, we decided to reduce the dose of EVR + reduced-TAC therapy to explore the appropriate dose for 30% partial LT.

We chose 1.5 mg/kg as the dose of combined TAC, the minimum dose needed to suppress TCMR for TAC monotherapy **(Supplemental Figures 2 and 3).** And then, the dose of combined EVR was gradually reduced from 1.3 mg/kg to 0.8 mg/kg **(Table 2)**.

In the EVR0.8 + TAC1.5 group, the 14-day survival rates were increased to 75% (n=8; **Figure 3**). Of the total 20 rats including those that were sacrificed on POD3 and POD5 (n=6 for each), four (20%) still showed abscess formation or bacterial colonies in the liver grafts, while mild TCMR (RAI: P1, B1, V1) was observed in one other rat within the same dose group (**Table 2 and Figure 4B**). Therefore, we could not reduce the doses any further. By contrast, in the EVR0.8 + TAC1.5 group, the trough concentrations in rats without abscess, bacterial colonies, or TCMR were close to the target values. The serum levels of BUN in the five rats that survived until POD14 without abscess, bacterial colonies, or TCMR, were significantly lower than those in the TAC6.5 group. The serum levels of AST, ALT, and T-Bil were comparable with the TAC6.5 group (**Supplemental**

Figure 4). Patency of the hepatic artery was confirmed in all rats that survived until POD14. Therefore, we considered the EVR0.8 + TAC1.5 group as an acceptable dose group to evaluate outcomes after 30% partial LT.

All rats in the TAC6.5 group exhibited neither infection nor TCMR in the liver grafts. The trough concentrations were close to the target values **(Table 2)**. We designated the TAC6.5 group as a standard dose TAC monotherapy group. The mean scores for total RAI in the liver grafts in the EVR2.5 group were 5.4±0.5, indicative of moderate to severe TCMR, although the trough concentrations were much higher than the target values.

No wound complications, including incisional hernia, were observed in any of the experiments.

3.4 Experiment 2-2: Liver regeneration after 30% partial LT

We evaluated liver regeneration after 30% partial LT in the EVR0.8 + TAC1.5 group, in addition to the EVR1.3 + TAC3.0 group. The weight ratio of the liver graft to the initial whole liver was defined by the following formula: (liver graft weight/recipient body weight at sacrifice)/ (initial whole liver weight/recipient body weight at pre-transplant) × 100 (%).

The liver graft weights on POD3 in the EVR0.8 + TAC1.5 and the EVR1.3 + TAC3.0

groups were restored only up to 59.3±3.4% and 59.2±4.0% respectively, compared with the initial whole liver, demonstrating a significant decrease compared with the TAC6.5 group (72.9±2.2%; both P<0.001) (**Figure 5A**). The liver graft weights in the EVR0.8 + TAC1.5 group did not reach those of the TAC6.5 group, even on POD14 (88.1±3.1% *vs.* 95.5±4.2%; P=0.01).

The Ki-67 labeling indices on POD3 in the EVR1.3 + TAC3.0 group were significantly lower than those in the TAC6.5 group (53.8±11.1% *vs.* 78.6±4.0%; P=0.03), while those in the EVR0.8 + TAC1.5 group (66.4±12.6%) approached the level of the TAC6.5 group **(Figure 5B)**. Representative liver sections stained by Ki-67 on POD3 are shown in **Figure 5C**.

3.5 Experiment 3: 14-day survival study and histological findings in liver grafts after whole LT

Only EVR + reduced-TAC therapy was performed in Experiment 3 (whole LT) because EVR monotherapy failed to suppress TCMR in Experiment 2. Since the EVR0.8 + TAC1.5 group was estimated as a low dose group for whole LT because of larger grafts, we chose the EVR1.3 + TAC3.0 group. All rats in the EVR1.3 + TAC3.0 group survived until POD14, and all had trough concentrations of EVR (8.2±2.9 ng/mL) and TAC

(1.8±0.8 ng/mL) close to the target levels on POD14 (n=5). The mean total RAI in the liver grafts was 1.0±1.2, with no TCMR. No abscess formation or bacterial colonies were observed in any liver graft. Patency of the hepatic artery after whole LT was confirmed on POD14 in all rats. Therefore, the EVR1.3 + TAC3.0 group was considered appropriate for whole LT.

4. DISCUSSION

The results of this study suggest that caution should be exercised with respect to very early introduction of EVR after partial LT. EVR + reduced-TAC therapy promoted less liver regeneration than standard dose TAC monotherapy. The survival rate for EVR + reduced-TAC therapy in the partial LT experiment was 75%, which was not as high as the value of 100% observed for standard dose TAC monotherapy, and neither bacterial infection nor rejection could be prevented. EVR monotherapy did not suppress rejection. By contrast, the survival rate for EVR + reduced-TAC therapy in the whole LT experiment was 100%, and no infection or rejection was observed.

There is interest in whether early introduction of EVR within 30 days after partial LT, such as LDLT, is safe and leads to preservation of renal function, as reported for deceased-donor LT.[6,9-13] Jeng et al. reported 43 cases in which introduction of EVR on

POD12 (mean; range 4–20) after LDLT was safe and feasible, but liver regeneration was not evaluated.[26] The antiproliferative effect of EVR suggests a negative impact on liver regeneration, but no previous reports have carefully evaluated the effects of EVR on liver regeneration.

In the present study, we found that EVR delayed liver regeneration. This result is consistent with that reported for sirolimus.[27,28] Toso et al. reported that introduction of sirolimus immediately after LDLT inhibited hepatocyte proliferation.[29] Ideally, liver regeneration should be evaluated in the absence of liver damage (e.g., infection or rejection) because liver weight and Ki-67 can be affected by liver damage. Therefore, we explored the appropriate doses that would suppress both infection and rejection. Due to more intense liver injury after partial LT (mainly liver ischemia and reperfusion injury), the doses of EVR + reduced-TAC therapy determined in the hepatectomy experiment required reduction in the partial LT experiment. Although some rats with infection or rejection were included because of the difficulty in managing doses of EVR and TAC in the partial LT experiment, the results were consistent with those of the hepatectomy experiments, in which normal liver histology was confirmed, in terms of delayed liver regeneration. Liver regeneration was slightly delayed in rats that underwent partial LT compared with rats that underwent hepatectomy in the early phase after surgery,

regardless of the immunosuppressive regimens, with this delay possibly due to liver ischemia and reperfusion injury. Liver regeneration following hepatectomy plus EVR + reduced-TAC therapy reached almost the same level as for control and standard dose TAC monotherapy. However, the first few days after LT are a very critical time for the liver graft since it must meet the recipient's metabolic demands while not only withstanding rejection, but also regenerating; this is particularly true when the transplanted graft is small for the size of the recipient. Therefore, even if liver regeneration was not suppressed continually, a delay in the early phase would adversely affect post-transplant recovery. In this study, we assumed that the combined use of EVR and TAC further increased the metabolic demands on the liver and imposed a heavy burden under conditions of delayed liver regeneration. Consequently, blood concentrations of EVR and TAC became unstable, combined with pharmacological interactions between EVR and TAC, which led to different outcomes within the same dose group with respect to infection and rejection.[23]

As a result, survival for EVR + reduced-TAC therapy in the partial LT experiment was lower than standard dose TAC monotherapy. Abnormal findings in the liver graft would have contributed directly to death because we identified either infection or rejection in almost all autopsied liver grafts, while other abnormal findings leading to death, such as

intra-abdominal abscess and bleeding, bowel obstruction, and wound complications, were not identified at autopsy. The survival rate for EVR monotherapy was 100% in the partial LT experiment, overcoming its negative effect on liver regeneration; however, rejection was not suppressed. In the H2304 trial, randomization to EVR monotherapy was terminated prematurely due to a higher rate of TCMR.[1]

These rat experiments highlight the difficulty in managing doses of EVR and TAC to suppress both infection and rejection following very early introduction of EVR with reduced-TAC after partial LT. These difficulties are caused by the negative effect of EVR on liver regeneration. From this perspective, it may be appropriate to introduce EVR later than 30 days post-LT, a strategy practiced widely today. However, these difficulties may be overcome by intense dose control of EVR and TAC. In the partial LT experiment, the Ki-67 labeling index for EVR + reduced-TAC therapy, which was used to evaluate hepatocyte proliferation, approached the level of standard dose TAC monotherapy by adjusting the doses carefully. Rats without infection or rejection that underwent EVR + reduced-TAC therapy exhibited better renal function than rats receiving standard dose TAC monotherapy. Therefore, it may be a preferable to measure trough concentrations daily and fine-tuning dosage in addition to selecting larger grafts if EVR is introduced immediately after partial LT.

By contrast, all rats in the whole LT experiment survived without infection or rejection, despite receiving the same dose used for EVR + reduced-TAC therapy, which resulted in the lowest survival rates in the partial LT experiment. This result also suggests that the negative effect of EVR on liver regeneration would have contributed to the poorer outcomes after partial LT, which required liver regeneration to sustain life. In LT, the ideal doses of immunosuppressive drugs are those that suppress both infection and rejection, but the ranges of EVR and TAC doses required to prevent these negative outcomes were narrow after partial LT than after whole LT. There is little doubt that graft size has a significant impact on post-LT outcomes.

This study has several limitations. The experimental model did not completely simulate the clinical setting. First, recipient rats had no liver cirrhosis or renal dysfunction prior to LT. Second, a fixed dose of immunosuppressive drugs was administered daily. Nevertheless, unlike for EVR + reduced-TAC therapy after partial LT, dose adjustment was unnecessary for TAC monotherapy following partial LT, and for EVR + reduced-TAC therapy following whole LT. Third, consideration should be given to species-specific differences in drug susceptibility, the dose of immunosuppressive drugs required to suppress rejection, and the size of the liver graft required for survival. Fourth, we did not perform any LT using grafts other than 30% or whole liver grafts.

In conclusion, the very early introduction of EVR, with or without reduced-TAC, for partial LT delayed liver regeneration and made it difficult to manage these doses needed for suppression of both infection and rejection. By contrast, EVR with reduced-TAC could be introduced safely very early after whole LT.

ACKNOWLEDGMENTS

The authors are grateful to the Center for Anatomical, Pathological, and Forensic Medical Researches, Graduate School of Medicine, Kyoto University, for preparing the microscope slides.

CONFLICT OF INTEREST

Author M.S. was supported by “Grant-in-Aid for Early-Career Scientists (Grant Number JP20K17581)” from the Japan Society for the Promotion of Science, Tokyo, Japan. The funding source has no role in the design, practice or analysis of this study.

REFERENCES

1. De Simone P, Nevens F, De Carlis L, Metselaar HJ, Beckebaum S, Saliba F, et al; H2304 Study Group. Everolimus with reduced tacrolimus improves renal function in de novo liver transplant recipients: a randomized controlled trial. Am J Transplant. 2012;12:3008-3020.
2. Jeng LB, Lee SG, Soin AS, Lee WC, Suh KS, Joo DJ, et al. Efficacy and safety of everolimus with reduced tacrolimus in living-donor liver transplant recipients: 12-month results of a randomized multicenter study. Am J Transplant. 2018;18:1435-1446.
3. Cholongitas E, Mamou C, Rodríguez-Castro KI, Burra P. Mammalian target of rapamycin inhibitors are associated with lower rates of hepatocellular carcinoma recurrence after liver transplantation: a systematic review. Transpl Int. 2014;27:1039-1049.
4. Nitta H, Younès A, El-Domiaty N, Karam V, Sobesky R, Vibert E, et al. High trough levels of everolimus combined to sorafenib improve patients survival after hepatocellular carcinoma recurrence in liver transplant recipients. Transpl Int. 2021;34:1293-1305.

5. Saliba F, Dharancy S, Salamé E, Conti F, Eyraud D, Radenne S, et al. Time to Conversion to an Everolimus-Based Regimen: Renal Outcomes in Liver Transplant Recipients From the EVEROLIVER Registry. Liver Transpl. 2020;26:1465-1476.
6. Rodríguez-Perálvarez M, Guerrero M, Barrera L, Ferrín G, Álamo JM, Ayllón MD, et al. Impact of Early Initiated Everolimus on the Recurrence of Hepatocellular Carcinoma After Liver Transplantation. Transplantation. 2018;102:2056-2064.
7. Yan X, Huang S, Yang Y, Lu Z, Li F, Jiang L, et al. Sirolimus or Everolimus Improves Survival After Liver Transplantation for Hepatocellular Carcinoma: A Systematic Review and Meta-Analysis. Liver Transpl. 2022;28:1063-1077.
8. Willuweit K, Heinold A, Rashidi-Alavijeh J, Heinemann FM, Horn PA, Paul A, et al. Immunosuppression with mTOR inhibitors prevents the development of donor-specific antibodies after liver transplant. Clin Transplant. 2017;31:e12974.
9. Masetti M, Montalti R, Rompianesi G, Codeluppi M, Gerring R, Romano A, et al. Early withdrawal of calcineurin inhibitors and everolimus monotherapy in de novo liver transplant recipients preserves renal function. Am J Transplant. 2010;10:2252-2262.
10. Gastaca M, Bilbao I, Jimenez M, Bustamante J, Dopazo C, Gonzalez R, et al. Safety and Efficacy of Early Everolimus When Calcineurin Inhibitors Are Not

Recommended in Orthotopic Liver Transplantation. Transplant Proc. 2016;48:2506-2509.

11. Cillo U, Saracino L, Vitale A, Bertacco A, Salizzoni M, Lupo F, et al. Very Early Introduction of Everolimus in De Novo Liver Transplantation: Results of a Multicenter, Prospective, Randomized Trial. Liver Transpl. 2019;25:242-251.
12. Nashan B, Schemmer P, Braun F, Schlitt HJ, Pascher A, Klein CG, et al; Hephaistos Study Group. Early Everolimus-Facilitated Reduced Tacrolimus in Liver Transplantation: Results From the Randomized HEPHAISTOS Trial. Liver Transpl. 2022;28:998-1010.
13. McKenna GJ. The HEPHAISTOS Trial and mTOR Inhibitors in Liver Transplantation - Hiding in Plain Sight. Liver Transpl. 2022;28:925-927.
14. Asrani SK, Wiesner RH, Trotter JF, Klintmalm G, Katz E, Maller E, et al. De novo sirolimus and reduced-dose tacrolimus versus standard-dose tacrolimus after liver transplantation: the 2000-2003 phase II prospective randomized trial. Am J Transplant. 2014;14:356-366.
15. Chapman TM, Perry CM. Everolimus. Drugs. 2004;64:861-872.
16. Kajiwara M, Masuda S. Role of mTOR Inhibitors in Kidney Disease. Int J Mol Sci. 2016;17:975.

17. Higgins GM, Anderson RM. Experimental pathology of the liver: restoration of the liver the white rat following partial surgical removal. Arch Pathol. 1931;12:186-202.

18. Kamada N, Calne RY. Orthotopic liver transplantation in the rat. Technique using cuff for portal vein anastomosis and biliary drainage. Transplantation. 1979;28:47-50.

19. Liu T, Freise CE, Ferrell L, Ascher NL, Roberts JP. A modified vascular "sleeve" anastomosis for rearterialization in orthotopic liver transplantation in rats. Transplantation. 1992;54:179-180.

20. Yagi S, Nagai K, Kadaba P, Afify M, Teramukai S, Uemoto S, et al. A novel organ preservation for small partial liver transplantations in rats: venous systemic oxygen persufflation with nitric oxide gas. Am J Transplant. 2013;13:222-228.

21. Hirata M, Yagi S, Ito T, Masano Y, Okumura S, Yao S, et al. Stepwise approach for acquisition of microsurgical skills through rat orthotopic liver transplantation experiments. Eur Surg Res. 2022 Nov 15. doi: 10.1159/000528092. Epub ahead of print.

22. Cillo U, De Carlis L, Del Gaudio M, De Simone P, Fagiuoli S, Lupo F, et al. Immunosuppressive regimens for adult liver transplant recipients in real-life practice:

consensus recommendations from an Italian Working Group. Hepatol Int. 2020;14:930-943.

23. Piao SG, Lim SW, Doh KC, Jin L, Heo SB, Zheng YF, et al. Combined treatment of tacrolimus and everolimus increases oxidative stress by pharmacological interactions. Transplantation. 2014;98:22-28.

24. Demetris AJ, Bellamy C, Hübscher SG, O'Leary J, Randhawa PS, Feng S, et al. 2016 Comprehensive Update of the Banff Working Group on Liver Allograft Pathology: Introduction of Antibody-Mediated Rejection. Am J Transplant. 2016;16:2816-2835.

25. Yamamoto C, Yagi S, Hori T, Iida T, Taniguchi K, Isaji S, et al. Significance of portal venous VEGF during liver regeneration after hepatectomy. J Surg Res. 2010;159:e37-43.

26. Jeng LB, Thorat A, Hsieh YW, Yang HR, Yeh CC, Chen TH, et al. Experience of using everolimus in the early stage of living donor liver transplantation. Transplant Proc. 2014;46:744-748.

27. Francavilla A, Starzl TE, Scotti C, Carrieri G, Azzarone A, Zeng QH, et al. Inhibition of liver, kidney, and intestine regeneration by rapamycin. Transplantation. 1992;53:496-498.

28. Palmes D, Zibert A, Budny T, Bahde R, Minin E, Kebschull L, et al. Impact of rapamycin on liver regeneration. Virchows Arch. 2008;452:545-557.

29. Toso C, Patel S, Asthana S, Kawahara T, Girgis S, Kneteman NN, et al. The impact of sirolimus on hepatocyte proliferation after living donor liver transplantation. Clin Transplant. 2010;24:695-700.

FIGURE LEGENDS

Figure 1. Schema of LT models and experimental design.

(A) Schema of allogeneic 30% partial LT and whole LT models.

The 30% partial or whole liver grafts from DA donors were transplanted orthotopically into LEW recipients.

(B) Experimental design.

Scheme showing 70% hepatectomy (Experiment 1), 30% partial LT (Experiment 2), and whole LT (Experiment 3). Immunosuppressive drugs were administered by oral gavage within 3 hours after hepatectomy or LT and once daily every morning thereafter until sacrifice. Liver regeneration, T cell-mediated rejection, and survival were evaluated using five different dose groups.

DA, Dark Agouti; EVR, everolimus; Exp, experiment; LEW, Lewis; LT, liver transplantation; TAC, tacrolimus.

Figure 2. Liver regeneration after 70% hepatectomy (Experiment 1).

(A) Liver regeneration rate (%) ($^{*}P<0.05$, n=4 for each).

(B) Quantification of the Ki-67 labeling index (%) ($^{*}P<0.05$, n=4 for each).

Black bar = average marker. EVR, everolimus; POD, post-operative day; TAC,

tacrolimus.

Figure 3. Survival study after 30% partial LT (Experiment 2).

The 14-day survival rates in the EVR1.3 + TAC3.0 and the EVR0.8 + TAC1.5 groups were 20% and 75%, respectively ($P<0.001$, n=3–8 for each).

EVR, everolimus; LT, liver transplantation; POD, post-operative day; TAC, tacrolimus.

Figure 4. Histological findings in liver grafts after sacrifice or at autopsy after 30% partial LT (Experiment 2).

(A) Representative autopsied liver sections from the EVR1.3 + TAC3.0 group.

H&E staining (left, original magnification, ×200) shows hepatocyte necrosis, abscess formation, and bacterial colonies (black arrow). Giemsa staining (right, original magnification, ×1000) reveals sinusoidal bacterial colonies (black arrows).

(B) Liver sections from one rat in the EVR0.8 + TAC1.5 group, which was sacrificed on POD5.

H&E staining (original magnification, ×200) shows lymphocytic inflammation and infiltration (white arrows) in the portal tracts (left) and perivenular areas (right), indicative of mild rejection.

EVR, everolimus; H&E, hematoxylin-eosin; LT, liver transplantation; POD, post-operative day; TAC, tacrolimus.

Figure 5. Liver regeneration after 30% partial LT (Experiment 2).

(A) Ratio of liver graft weight to initial whole liver weight (*P<0.05; n=3–6 for each).

(B) Quantification of the Ki-67 labeling index (%) (*P<0.05; n=3–6 for each).

(C) Representative liver sections stained for Ki-67 on POD3 (original magnification, ×200).

Black bar = average marker. EVR, everolimus; POD, post-operative day; TAC, tacrolimus.

A LIST OF SUPPORTING INFORMATION

1. **Supplemental Methods**

2. **Supplemental Figure**

Supplemental Figure 1. Serum levels of BUN and Cre on POD14 after 70% hepatectomy (Experiment 1).

Serum levels of BUN and Cre were evaluated between four groups excluding the control group ($^{*}P<0.05$; n=4 for each). Black bar = average marker. BUN, blood urea nitrogen; Cre, creatinine; EVR, everolimus; POD, post-operative day; TAC, tacrolimus.

Supplemental Figure 2. Survival study for TAC monotherapy after 30% partial LT (Experiment 2).

All rats survived until POD14, except for two in the TAC0.3 group (P = 0.13; n=2–3 for each). LT, liver transplantation; POD, post-operative day; TAC, tacrolimus.

Supplemental Figure 3. Total RAI score in liver grafts after sacrifice or at autopsy for TAC monotherapy after 30% partial LT (Experiment 2).

Total RAI score in liver grafts from rats sacrificed on POD14, and in the autopsied

liver grafts before POD14 (*P<0.05; n=2–3 for each). LT, liver transplantation; POD, post-operative day; RAI, rejection activity index; TAC, tacrolimus.

Supplemental Figure 4. Blood biochemistry on POD14 after 30% partial LT (Experiment 2).

Serum levels of AST, ALT, T-Bil, BUN, and Cre in the rats that survived until POD14 were evaluated (*P<0.05; n=5 for each). One dying rat on POD14 in the EVR0.8 + TAC1.5 group was excluded.

Black bar = average marker. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; Cre, creatinine; EVR, everolimus; LT, liver transplantation; POD, post-operative day; TAC, tacrolimus; T-Bil, total bilirubin.

Table 1. Whole blood EVR and TAC trough concentrations after 70% hepatectomy (Experiment 1)

Trough concentration (ng/mL)		EVR 1.3 (n=4 each)	EVR 2.5 (n=4 each)	TAC 6.5 (n=4 each)	EVR 1.3+TAC 6.5 (n=4 each; (※ n=2))	EVR 1.3+TAC 3.0 (n=4 each)	EVR 0.8+TAC 1.5 (n=4 each)
EVR	POD 1	5.6±1.5	6.8±3.3	None	7.5±3.5	4.0±2.2	3.1±1.6
	POD 3	4.9±1.6	9.6±1.4[a]	None	14.7±1.9[a,b,e]	8.3±1.4[a]	3.9±0.6[b,d,e]
	POD 7	4.8±0.9	9.8±0.8	None	22.1±9.1[a,b]	8.6±0.6[d]	4.6±0.6[d]
	POD 14	5.9±1.0	9.8±0.6	None	39.5※	13.2±2.0	3.8±0.7[d,e]
TAC	POD 1	None	None	9.2±3.5	11.5±2.1	2.6±0.8[c,d]	0.9±0.2[c,d]
	POD 3	None	None	11.8±2.7	10.2±3.2	2.6±0.7[c,d]	0.9±0.2[c,d]
	POD 7	None	None	10.4±1.5	21.6±11.5	2.4±0.7	0.6±0.3[d]
	POD 14	None	None	6.4±0.8	47.8※	2.5±0.5	0.5±0.0[c,d]

Values are presented as the mean ± standard deviation.

※ Two rats in the EVR 1.3+TAC 6.5 group died

a: P<0.05 versus the EVR 1.3 group

b: P<0.05 versus the EVR 2.5 group

c: P<0.05 versus the TAC 6.5 group

d: P<0.05 versus the EVR 1.3+TAC 6.5 group

e: P<0.05 versus the EVR 1.3+TAC 3.0 group

EVR, everolimus; POD, post-operative day; TAC, tacrolimus

Table 2. Outcomes of all groups tested in the 30% partial LT experiment (Experiment 2)

		Trough concentration (ng/mL)		Histological findings in liver grafts			
		EVR	TAC	Abscess formation/ Bacterial colonies		TCMR	
EVR 1.3+TAC 3.0 (n=15)	POD 3	13.1±2.2	8.9±1.8	1/5	Total 8/15	0/5	Total 0/15
	POD 5*	20.9±16.4	21.2±30.4	5/7		0/7	
	POD 6*	None	None	1/1		0/1	
	POD 9*	None	None	1/1		0/1	
	POD 14	10.5	2.8	0/1		0/1	
EVR 1.3+TAC 1.5 (n=2)	POD 7	9.3	1.6	0/1	Total 0/2	0/1	Total 0/2
	POD 14	18.2	2.2	0/1		0/1	
EVR 1.0+TAC 1.5 (n=1)	POD 6*	None	None	1/1	Total 1/1	0/1	Total 0/1
EVR 0.8+TAC 1.5 (n=20)	POD 3	5.7±1.3	3.7±1.8	1/6	Total 4/20	0/6	Total 1/20
	POD 5	4.7±1.1	1.5±0.9	0/6		1/6	
	POD 7*	None	None	2/2		0/2	
	POD 14	5.6±0.4	0.9±0.1	1/6		0/6	
TAC 6.5 (n=11)	POD 3	None	15.8±3.2	0/3	Total 0/11	0/3	Total 0/11
	POD 5	None	7.4±2.4	0/3		0/3	

	POD 14	None	12.0±5.9	0/5		0/5	
EVR 2.5 (n=5)	POD 14	21.0±3.7	None	0/5	Total 0/5	5/5	Total 5/5

Values are presented as the mean ± standard deviation.

* Autopsied cases are included.

EVR, everolimus; POD, post-operative day; TAC, tacrolimus; TCMR, T cell-mediated rejection; RAI, rejection activity index

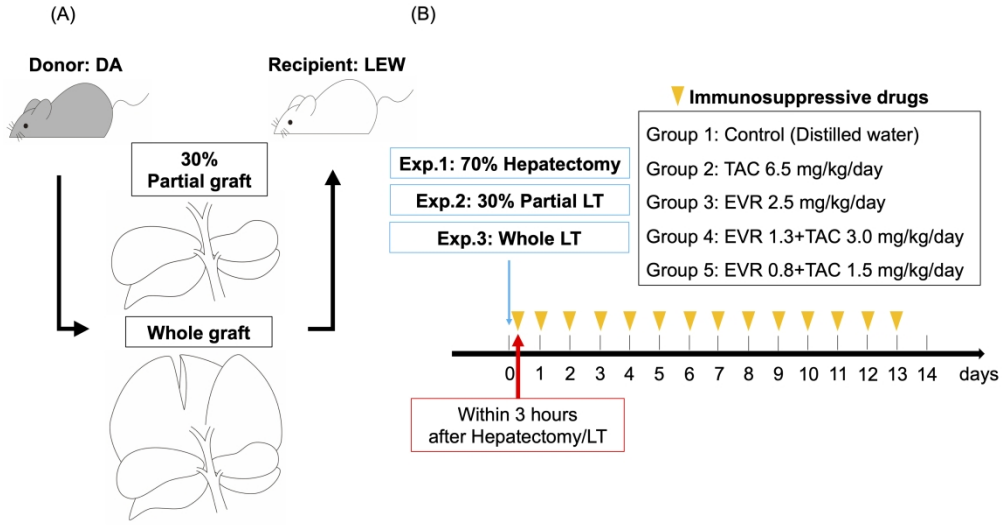

Figure 1

1411x793mm (72 x 72 DPI)

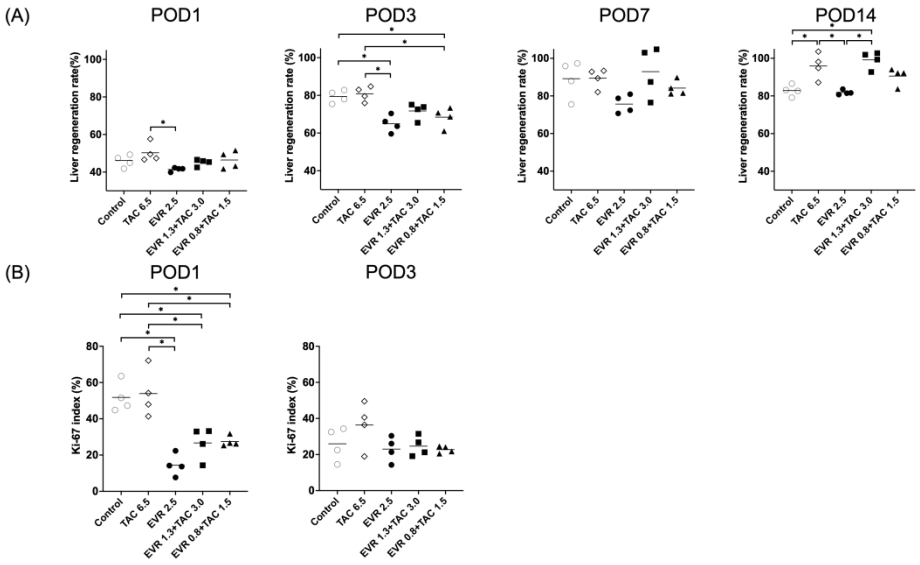

Figure 2

1411x793mm (72 x 72 DPI)

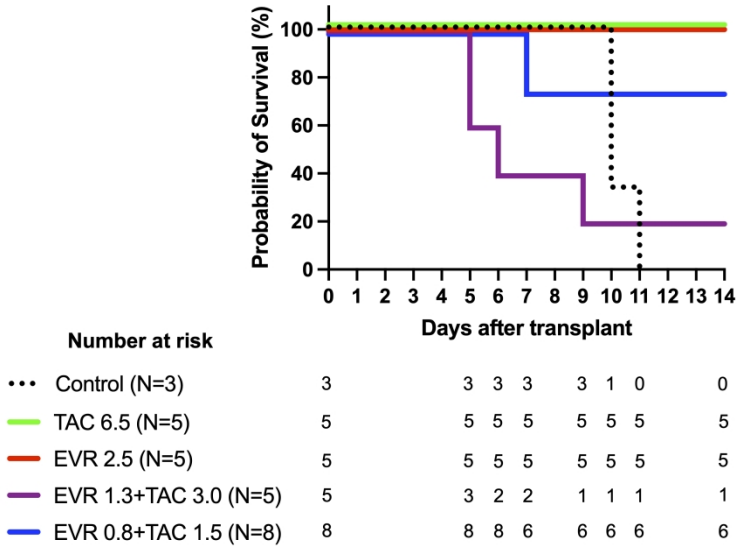

Figure 3

1411x793mm (72 x 72 DPI)

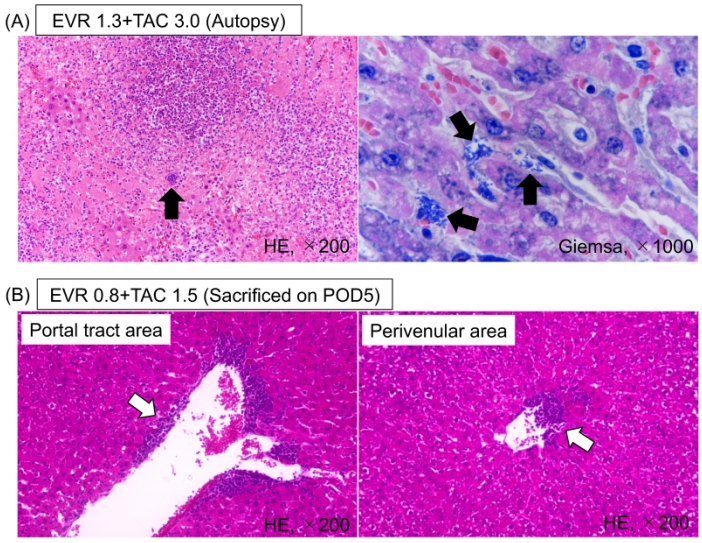

Figure 4

338x190mm (565 x 565 DPI)

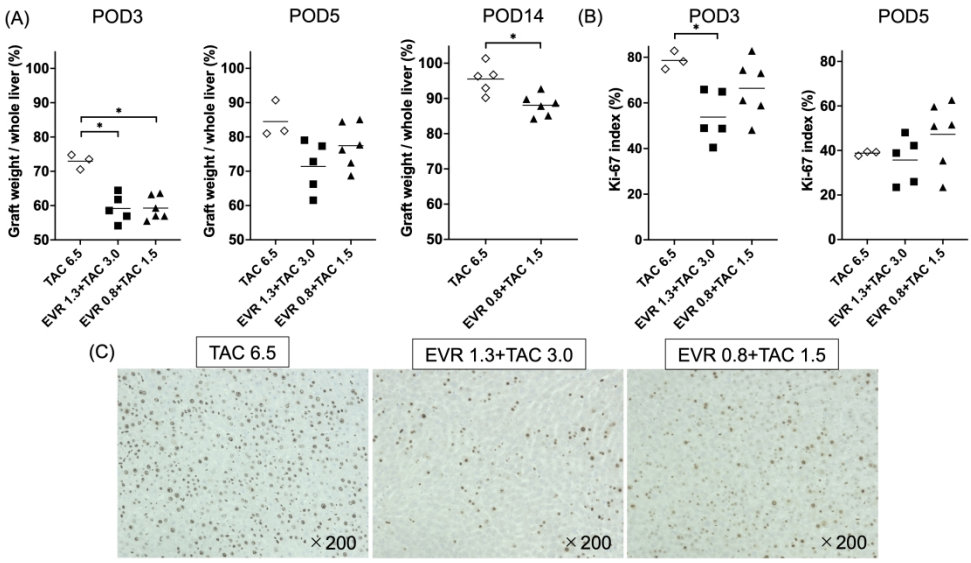

Figure 5

1411x793mm (72 x 72 DPI)