Suppression of Hepatocellular Carcinoma by Mycophenolic Acid in Experimental Models and in Patients

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Background. Tumor recurrence is a major complication following liver transplantation (LT) as treatment for hepatocellular carcinoma (HCC). Immunosuppression is an important risk factor for HCC recurrence, but conceivably may depend on the type of immunosuppressive medication. Mycophenolic acid (MPA) is a currently widely used immunosuppressant. This study investigated the effects of MPA on HCC. **Methods.** Three human HCC cell lines and organoids from mouse primary liver tumor were used as experimental models. MTT, Alamar Blue assay, cell cycle analysis, colony formation, and [3H]-thymidine assays were performed. An LT database was used for retrospective analysis of the effect of mycophenolate mofetil, the prodrug of MPA, on HCC recurrence. **Results.** With clinically achievable concentrations, MPA effectively inhibited HCC cell proliferation and single-cell colony-forming unit. In short-term experiments, MPA effectively elicited S phase arrest in HCC cell lines. In addition, the initiation and growth of liver tumor organoids were effectively inhibited by MPA. Most importantly, the use of mycophenolate mofetil in patients with HCC-related LT was significantly associated with less tumor recurrence and improved patient survival. **Conclusions.** MPA can specifically counteract HCC growth in vitro and tumor recurrence in LT patients. These results warrant prospective clinical trials into the role of MPA-mediated immunosuppression following LT of patients with HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide.[1] Surgical resection or liver transplantation (LT) is currently the only potentially curative treatment options. LT is particularly attractive because of the radical resection of the tumor achieved. Moreover, LT cures the underlying liver disease along with the replacement of the diseased liver that remains at risk for the development of new malignant lesions when simple tumor resection is executed. However, tumor recurrence is a common threat for the success of both surgical resection and LT.[2] A unique risk factor strongly associated with recurrence in LT patients is the universal use of immunosuppressants after transplantation, which is to prevent graft rejection[3-5] but concomitantly hampers anticancer immunosurveillance.

Importantly, immunosuppression involves inhibition of immune cell proliferation, and thus such therapy might

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have direct effects on the cancerous compartment as well. Besides a general impairment of the immunosurveillance system, different types of immunosuppressants could thus directly affect the malignancy process independent of the host immunity.[4,6-8] Current research efforts in this respect are mainly focused on the mammalian target of rapamycin (mTOR) inhibitors, including rapamycin (sirolimus) and everolimus.[9] They are thought to be the only class of immunosuppressive agents that may reduce HCC recurrence, and this notion is supported by several retrospective and meta-analysis studies.[10-12] However, these studies do not provide firm evidence to establish superiority of mTOR inhibitors on HCC recurrence in comparison to other types of immunosuppression.[13] In a recent prospective study, it has been shown that sirolimus in LT recipients with HCC does not improve long-term recurrence-free survival beyond 5 years, although a beneficial effect between 3 and 5 years after transplantation in subgroups was suggested.[14,15] Furthermore, higher rejection rates were reported for monotherapy of sirolimus or everolimus in HCC patients with LT.[16,17] The differential effects of mTOR inhibitors in patients are probably related to the heterogenicity of HCC.[18,19] It is unlikely that 1 immunosuppression protocol fits all cases. Therefore, the impact of other immunosuppressants also deserves to be carefully investigated to define appropriate immunosuppressive regimens for management of HCC recurrence after LT.

Mycophenolic acid (MPA) and its prodrug, mycophenolate mofetil (MMF), are currently widely used for prevention of allograft rejection because of lacking nephrotoxicity.[20] These drugs act through depletion of guanine nucleotide pools by inhibition of inosine monophosphate dehydrogenase (IMPDH), in particular the isoform 2 (IMPDH2).[21] This results in blockage of de novo guanine nucleotide synthesis and inhibition of lymphocyte proliferation.[20] Interestingly, MPA has been reported to be able to inhibit cancer cell proliferation in several experimental models of human solid tumors and hematological malignancies.[22-25] A large prospectively observational cohort study observed a tendency toward a lower risk of malignancy in MMF- versus non-MMF-treated renal transplanted patients.[26] However, this class of immunosuppressant has not been extensively studied in the setting of HCC recurrence after LT. This consideration inspired us to explore the effects and mechanism of action of MPA in experimental HCC models and HCC-related LT patients.

MATERIALS AND METHODS

Patient Information

An LT database established in our previous study[5] was used for retrospective analysis of the effect of MMF on HCC recurrence. This cohort included patients transplanted between October 1986 and December 2007 at the Erasmus Medical Centre, Rotterdam, the Netherlands. All patients declared that they did not object to the use of their data in the study. Retrospective analysis of clinical data was performed in accordance with the approval and guidelines of the Medical Ethical Committee of the Erasmus Medical Center. From this database, 44 of 385 LT patients were identified as HCC-related LT and thus subjected to the analysis in this study. Their clinical information was described in Table S1 (SDC, http://links.lww.com/TP/B692).

Reagents

Stocks of MPA (AMRESCO LLC, Cleveland, OH) were dissolved in dimethyl sulfoxide (DMSO). The final concentrations of DMSO were ≤0.1%. MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was purchased from Sigma-Aldrich. Matrigel was purchased from BD Bioscience. For the cytokines, B27 and N2 were purchased from Invitrogen; *N*-acetylcysteine, gastrin, and nicotinamide were purchased from Sigma-Aldrich; and epidermal growth factor (EGF), fibroblast growth factor 10 (FGF10), hepatocyte growth factor (HGF) were purchased from Peprotech Company.

Cell Culture

HCC cell lines, including HuH6, HuH7, and PLC/PFR/5, were grown in DMEM (GIBCO Life Technologies) and supplemented with 10% (v/v) fetal bovine serum (Hyclone Technologies), 100 U/mL of penicillin, and 100 μg/mL of streptomycin. All the cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. For the control groups in this study, equal volumes of PBS containing the same concentration of DMSO as in the drugs were added, which were also marked as MPA at the concentration of 0 μM.

Tumor Organoids Culture

Single cells were isolated from liver tumor tissues of mice by using digestion solution as our previous study.[27,28] Cells were mixed with matrigel, and then planted into 24-well plates in a 37°C incubator for 30 minutes. After matrigel formed, a solid gel, medium was added softly. Advanced DMEM/F12 (Invitrogen) works as the basic culture medium, supplemented with B27, N2, *N*-acetylcysteine, gastrin, nicotinamide, EGF, FGF10, HGF, and R-spondin1 (produced by 293T-H-RspoI-Fc cell line). During the first 3 days, Noggin and Wnt3a (produced by 293T-HA-Noggin and L-Wnt3a cell lines, respectively) were added. The medium was replaced every 3 days and passage was performed according to the growth of organoids.

MTT and Alamar Blue Assays

Cells were seeded in 96-well plates, at a concentration of 6×10^3 cells/well in 100 μL medium. All cells were incubated overnight to attach to the bottom of the wells and then treated with serial dilutions of MPA (3, 15, 30, and 60 μM). Cell viability was analyzed by adding 5 mg/mL MTT for 3 hours and then 150 μL DMSO per well. Absorbance was determined by using a spectrophotometric plate reader (Enzyme mark instrument, CytoFluor Series 4000, Perseptive Biosystems) at the wavelength of 490 nm.

Organoids were split in the ratio of 1:10 for daily culture and seeded in 24-well plates. MPA (3 μM and 15 μM) was added to the organoids from the initial day. At the third day, organoids were incubated with Alamar Blue (Invitrogen, 1:20 in DMEM) for 4 hours, and medium was collected for analysis of the metabolic activity of the organoids. Absorbance was determined by using a fluorescence plate reader (CytoFluor Series 4000, Perseptive Biosystems) at the excitation of 530/25 nm and emission of 590/35 nm. Each treatment condition was repeated 3 times and matrigel only was used as blank control.

Colony Formation Assay

Cells were procured and suspended in medium, then seeded into 6-well plates (1000 cells/well). Formed colonies were fixed by 70% ethanol and counterstained with hematoxylin and eosin after 2 weeks. Colony numbers were counted.

For single organoid formation, organoids were digested into single cells at first, and then the single living cells were further isolated by FACS sorter (Aria, BD Biosciences). Propidium iodide staining was performed to exclude dead cells. Single cells were mixed with matrigel and seeded in 24-well plates (100 cells/well) for organoids initiation. Single organoids were formed after 5 days, and the sizes and numbers of the organoids were calculated.

Analysis of Cell Cycle

Cells (5×10^5/well) were plated in 6-well plates and incubated overnight to attach the bottom, and then serial concentrations of MPA were added. After 48 hours, control and treated cells were trypsinized and washed with PBS and then fixed in cold 70% ethanol overnight at 4°C. The cells were washed twice with PBS and incubated with 20 μg/mL RNase A at 37°C for 30 minutes, and then with 50 μg/mL propidium iodide at 4°C for 30 minutes. The samples were analyzed immediately by FACS Calibur. Cell cycle was analyzed by using Flowjo 7.6 software.

T-cell Isolation and [3H]-Thymidine Assay

Peripheral blood mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque (Life Technologies). T cells were isolated with the Pan T-cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. Dynabeads coated with human T-activator CD3/CD28 antibodies (Life Technologies) were added at a cell:bead ratio of 20:1 T cells/well to stimulate T-cell expansion and activation. T cells were cultured in round-bottom 96-well plates at the concentration of 1×10^5 cells/well in 200 μL RPMI 1640 medium (GIBCO Life Technologies) supplemented with 10% FCS, at 37°C, and 5% CO_2, with or without compounds. After 3 days, T-cell proliferation was assessed by determination of [3H]-thymidine (Radiochemical Central, Little Chalfont, United Kingdom) incorporation, 0.5 μCi/well was added, and cultures were procured 18 hours later.

Statistical Analysis

Statistical analysis was performed by using chi-square test, nonparametric Mann-Whitney test, Cox regression analysis, and Kaplan-Meier survival analysis in IBM SPSS Statistical program (IBM Corporation, Armonk, NY). Mann-Whitney *U* test and *t* test were performed by using GraphPad InStat software (Graph Pad Software, Inc, San Diego, CA). *P* values <0.05 were considered as statistically significant.

RESULTS

Use of MMF Is Associated With Reduced HCC Recurrence and Improved Survival

We investigated the effect of MPA on the outcome of LT patients indicated by HCC in a prospectively collected LT cohort.[5] We have identified 44 of 385 patients with HCC-related LT. Twelve cases of these HCC patients were treated with immunosuppressive regimens containing MMF at any time during the follow-up and for any period, whereas 32 patients were treated with immunosuppressive regimens that did not contain MMF. There were no significant differences between these groups regarding patient characteristics, including age and sex, and regarding known prognostic factors of HCC recurrence after LT,[29] including the size of tumor, number of lesions, tumor differentiation stage, vascular invasion, level of α-fetoprotein before transplantation, and time of follow-up (Table 1).

However, only 1 of 12 patients (8.3%) in the MMF group developed recurrence, whereas 15 of 32 patients (46.9%) in the control group developed recurrence during follow-up. One patient died in MMF group (8.3%), but 18 patients died (56.3%) in the control group. Thus, the use of MMF was significantly associated with lower recurrence rates ($P < 0.05$; Table 1) and higher survival rates ($P < 0.01$; Table 1). Kaplan-Meier analysis confirmed

TABLE 1.

Patient characteristics according to MMF use

No.	Characteristics	MMF use		
		No (%/median)	Yes (%/median)	*P*[a]
1	Age	54.94	56.33	–
2	Sex (% male)	23/32 (71.9%)	10/12 (83.3%)	0.446
3	Recurrence	15/32 (46.9%)	1/12 (8.3%)	0.017*
4	Death	18/32 (56.3%)	1/12 (8.3%)	0.004**
5	Size of tumor (≥2 cm)[b]	18/32 (56.2%)	8/12 (66.7%)	0.542
6	No. of lesions (≥2)	20/31 (64.5%)	8/12 (66.7%)	0.898
7	Differentiation			
	Good	9/31 (29.0%)	3/11 (27.3%)	0.798
	Moderate-bad	22/31 (71.0%)	8/11 (72.7%)	0.789
8	Vasoinvasion	9/30 (30%)	1/11 (9.1%)	0.176
9	AFP (>25 μg/L) pretransplantation	11/20 (55%)	4/12 (33%)	0.248

[a]Categorized parameters were compared using Pearson chi-square test, and mean differences were tested using Mann-Whitney test.
[b]According to the Milan criteria, single lesion ≤5 cm or up to 3 individual lesions with none >3 cm.
AFP, α-fetoprotein; MMF, mycophenolate mofetil.
*$P < 0.05$; **$P < 0.01$.

that patients using MMF have significantly delayed HCC recurrence ($P \leq 0.05$; Figure 1A) and associated with better patient survival ($P < 0.05$; Figure 1B). Consistently, Cox regression analysis revealed that patients using MMF have a lower risk of fast recurrence (progression; HR, 0.169; 95% CI, 0.022–1.284; Figure 1C) and lower risk of demise (HR, 0.128; 95% CI, 0.017–0.967; Figure 1D). These results indicate that MMF use is associated with reduced HCC recurrence and improved survival in LT patients.

MPA Inhibited Cell Proliferation and Colony Unit Formation of Human HCC cells

To investigate whether MPA may directly affect the cellular physiology of HCC cells, the effects on cell proliferation and single-cell colony-forming unit (CFU) were evaluated in different HCC cell lines. Treatment of MPA inhibits cell proliferation in HuH6, HuH7, and PLC/PRF/5 cell lines at clinically relevant concentrations ($P < 0.001$; Figure 2A). In LT patients, MPA serum peak levels range from 2 to 30 μM, and the drug levels in liver will exceed those observed in serum due to accumulation.[30,31] Sorafenib, the Food and Drug Administration-approved anti-HCC drug, is a small inhibitor of several tyrosine protein kinases, including vascular endothelial factor (VEGFR), platelet-derived growth factor receptor (PDGFR), and Raf family kinases.[32] The potency of MPA was comparable to sorafenib, in particular at the concentration of 3 μM, although weaker than sorafenib at a higher concentration of 15 μM ($P < 0.01$; Figure S1A and B, SDC, http://links.lww.com/TP/B692). Surprisingly, the widely used mTOR inhibitor, rapamycin, did not show inhibitory effect on HCC cells in our experimental setting at clinically relevant or even higher concentrations (Figure S1C, SDC, http://links.lww.com/TP/B692).[33]

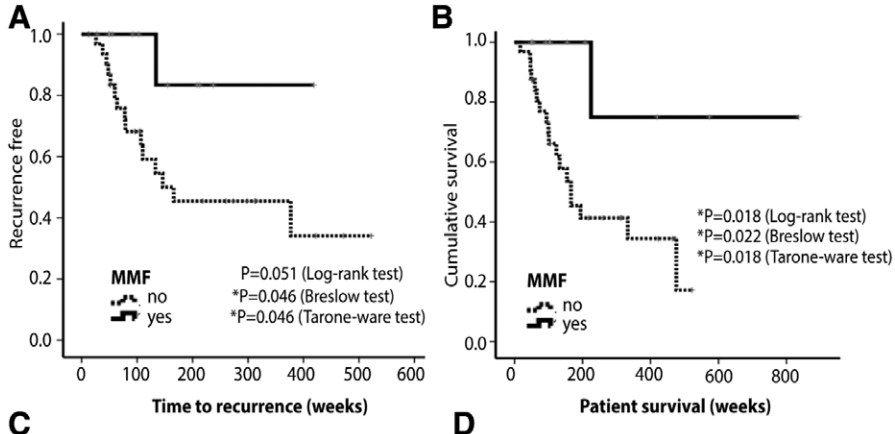
In apparent agreement, MPA profoundly inhibited the number of colonies formed in the CFU assay. It appears that even at a relatively low concentration of 3 μM, MPA already impeded colony formation (Figure 2B and C). HuH7 cells were more sensitive to MPA treatment compared with HuH6 and PLC/PRF/5 cells. In this cell model, 105.70 ± 13.90 colonies were formed in untreated cultures, but only 13.60 ± 11.25 colonies were formed in 15 μM MPA-treated group (mean ± SEM, n = 10, $P < 0.001$; Figure 2C). We concluded that MPA strongly interferes with HCC cell expansion in vitro.

MPA Effectively Inhibited the Initiation and Growth of Mouse Liver Tumor Organoids

Three-dimensional culture of primary tumor organoids has been recently demonstrated as advanced liver cancer models.[27,28,34] Therefore, we have investigated the effects of MPA on the initiation and growth of tumor organoids derived from primary mouse liver tumors. MPA effectively inhibited the growth of formed organoids shown by morphological appearance (Figure 3A). Alamar Blue assay demonstrated 79.03 ± 0.01% and 82.75 ± 0.01% inhibition at 3 μM and 15 μM, respectively (mean ± SEM, n = 3, $P < 0.001$; Figure 3B). Furthermore, MPA robustly inhibited the initiation of organoids from the dissociated single organoid cells (Figure 3C). The numbers of initiated organoids were 27.67 ± 4.51, 8 ± 1.00, and 4.67 ± 1.70 at 0 μM, 3 μM, and 15 μM of MPA, respectively (mean ± SEM, n = 3, $P < 0.001$; Figure 3D). The size of formed organoids was inhibited by 82.00 ± 0.08% and 89.09 ± 0.06% at 3 μM and 15 μM of MPA, respectively (mean ± SEM, n = 9, $P < 0.001$; Figure 3E).

Cell Cycling of HCC Cells Was Arrested at S Phase by MPA Treatment

To further understand how MPA acts on HCC cell growth, an assay for quantifying cell cycling was performed in HuH7 cells. Treatment of MPA dose dependently increased the proportion of S phase by 25.83 ± 0.20% and 131.42 ± 0.32% at the concentrations of 3 μM and 15 μM, respectively. This concomitantly decreased the proportion of cells in the G2/M phase by 67.82 ± 0.23% and 87.28 ± 0.09% at the concentrations of 3 and 15 μM, respectively (mean ± SEM, n = 3, $P < 0.05$; Figure 4).



C

MMF use	P-value	HR	95% CI for HR
yes	0.086	0.169	0.022-1.284

D

MMF use	P-value	HR	95% CI for HR
yes	*0.046	0.128	0.017-0.967

FIGURE 1. MMF use is significantly associated with better clinical outcome in HCC-related LT patients. Kaplan-Meier analysis (n = 44) revealed that patients using MMF display significantly longer times to HCC recurrence (*$P \leq 0.05$) (A) and have a better survival (*$P < 0.05$) (B). C, Consistently, Cox regression analysis showed that patients using MMF have a lower risk of fast recurrence (progression) (C) and lower risk of poor survival (*$P < 0.05$) (D). CI, confidence interval; HCC, hepatocellular carcinoma; HR, hazard ratio; LT, liver transplantation; MMF, mycophenolate mofetil.

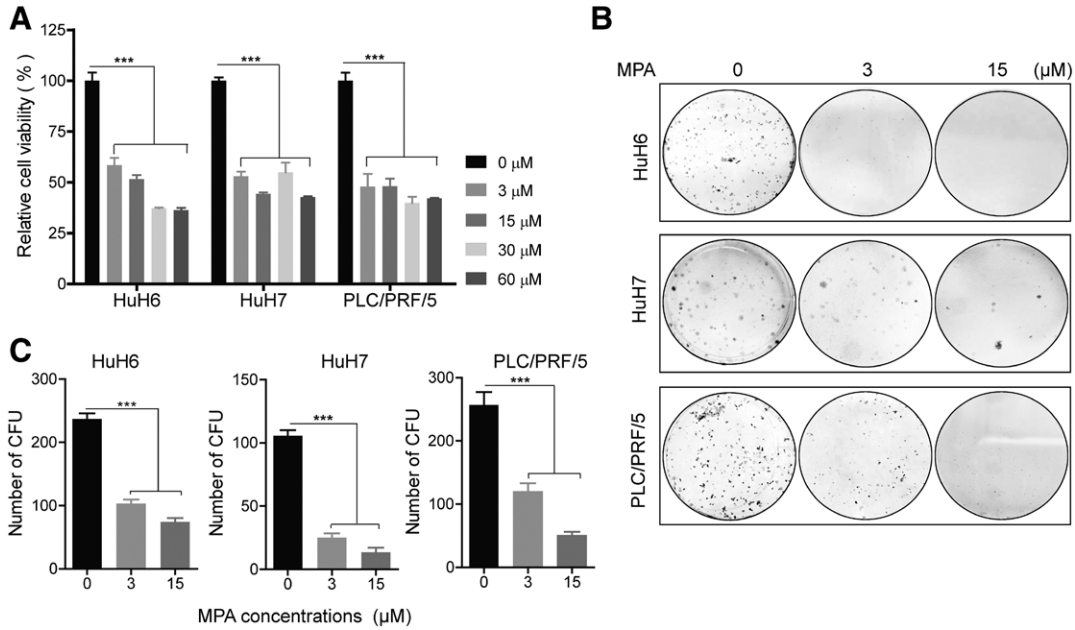


FIGURE 2. MPA inhibits cell growth in HCC cell lines. A, With clinically achievable concentrations, MPA potently inhibited cell proliferation, determined by MTT assay (mean ± SEM, n = 6, ***$P < 0.001$); (B) and (C), MPA inhibited the ability of colony formation in HuH6, HuH7, and PLC/PFR/5 cell lines, respectively (mean ± SEM, n = 9 or 10, respectively, ***$P < 0.001$). Results from at least 3 independent experiments are shown. CFU, colony-forming unit; HCC, hepatocellular carcinoma; MPA, mycophenolic acid; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; SEM, standard error of the mean.

These data suggested that MPA inhibits HCC cell growth by arresting the cell cycle.

Exogenous Nucleotide Supplementation Partially Counteracts the Antigrowth Effect of MPA

Depletion of intracellular nucleotide pool is the key immunosuppressive mechanism used by MPA to inhibit lymphocyte proliferation. Supplementation of exogenous guanosine nucleotide indeed partially counteracted the antiproliferative effects of MPA on HCC cell lines, but this effect is related to the cell type and dosage (Figure 5A). This effect was also observed in colony formation assay. The numbers of colonies were 102.17 ± 19.63, 31.17 ± 14.02, and 107.67 ± 27.73 in HuH6, HuH7, and PLC/PRF/5 cell lines with MPA (3 μM) treatment, respectively. Supplementation of exogenous guanosine nucleotide (25 μM) increased the colony numbers to 134.83 ± 29.49, 71.50 ± 9.95, and 145.67 ± 28.91 in HuH6, HuH7, and PLC/PRF/5 cell lines, respectively (mean ± SEM, n = 6, $P < 0.05$ or $P < 0.001$; Figure 5B and C). However, high doses

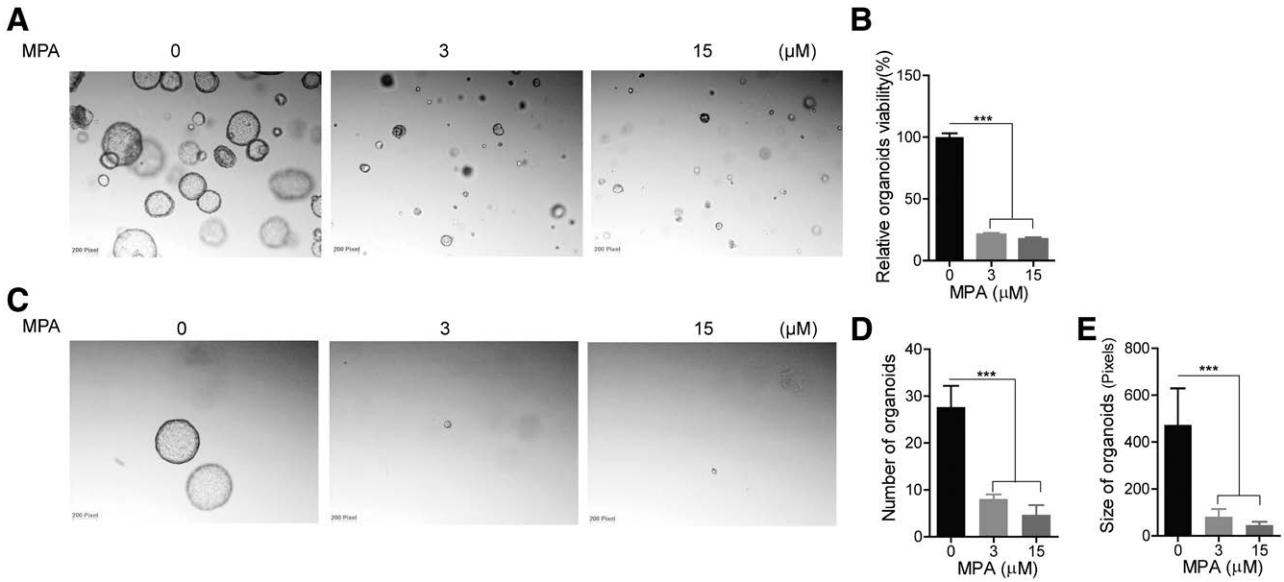


FIGURE 3. Mycophenolic acid (MPA) inhibits the initiation and growth of organoids established from mouse primary liver tumors. A, The appearance of organoids under 3-day MPA treatment; (B) MPA treatment significantly inhibited the growth of organoids, as determined by Alamar Blue assays after 3 days (mean ± SEM, n = 3, ***$P < 0.001$); (C) the appearance of single organoids expansion under 5-day MPA treatment; (D) the number of organoids (mean ± SEM, n = 3, **$P < 0.01$). E, The size of organoids after 5 days (mean ± SEM, n = 3, ***$P < 0.001$). Results from at least 3 independent experiments are shown. SEM, standard error of the mean.

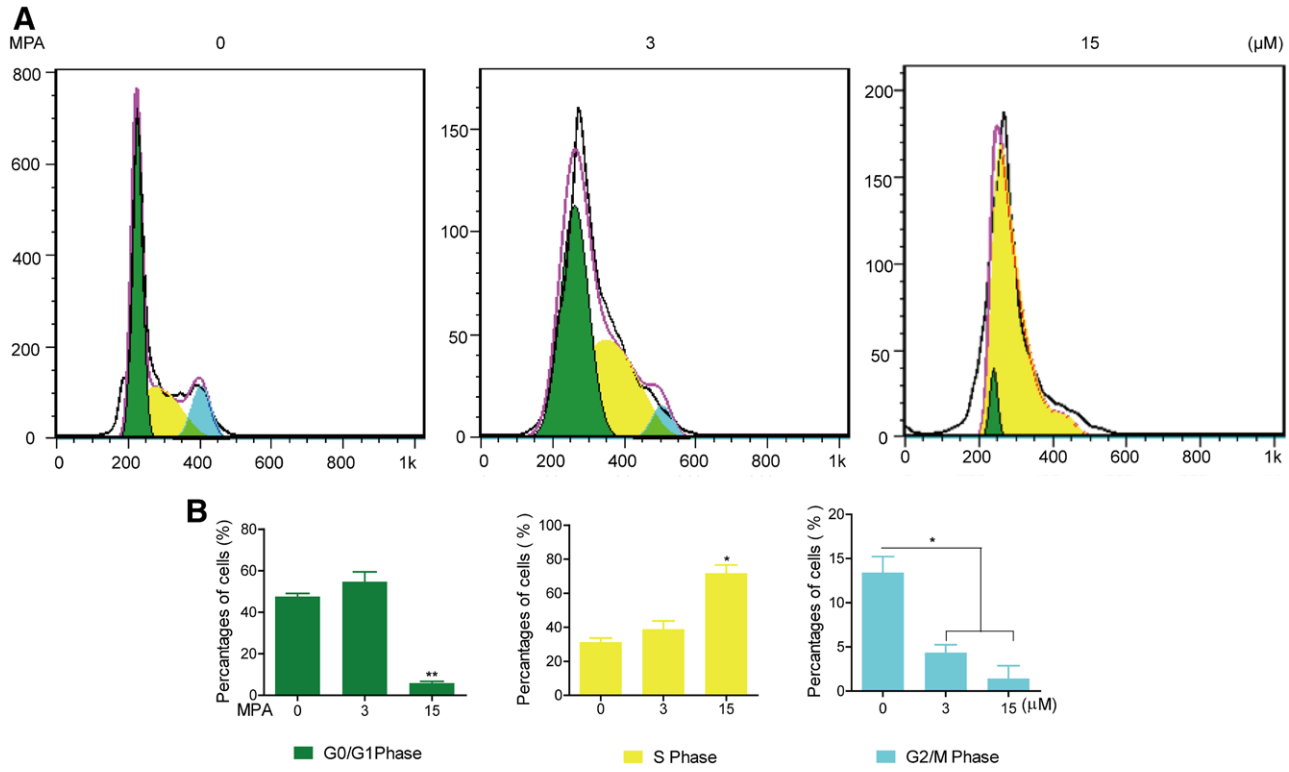


FIGURE 4. Mycophenolic acid (MPA) arrests cell cycling. A, HuH7 cells were arrested in the S phase by MPA treatment (FACS analysis); (B) Quantification of cell cycling analysis (mean ± SEM, n = 3, *P < 0.05, **P < 0.01). SEM, standard error of the mean.

of MPA outcompete exogenous guanosine nucleotides, especially in HuH7 and PLC/PRF/5 cells (Figure 5A–C).

New IMPDH Inhibitors Have Potential Immunosuppressive and Anti-HCC Properties

We explored the possibility to develop new IMPDH inhibitors exhibiting superior anti-HCC activity as compared to MPA but with comparable immunosuppressive activity, which may constitute improved treatment choices following HCC-indicated LT. Twenty-three IMPDH inhibitors were developed and profiled. Their immunosuppressive capability was evaluated in a T-cell proliferation assay. Fifteen of them were more potent than MPA in inhibiting T-cell proliferation after 72-hour treatment (mean ± SEM, n = 9, P < 0.01; Figure 6A). Intriguingly, 4 of these compounds (1351, 1353, 1382, and 1407) were identified as more potent inhibitors of HuH6 cells proliferation than MPA (mean ± SEM, n = 9, P < 0.05; Figure 6B). Collectively, 3 compounds (1351, 1353, and 1382) were found possessing both stronger immunosuppressive and antitumor activity (Figure 6C). Interestingly, 3 compounds significantly inhibit HuH6 cell proliferation (1393, 1400, and 1407) (compounds versus CTR, mean ± SEM, n = 9, P < 0.001) without affecting T-cell growth (Figure 6C), which suggests that these compounds may have potential as new generation of anti-HCC drugs in a nontransplant setting that does not require immunosuppression.

DISCUSSION

Although it is suspected that immunosuppressive medication following LT facilitates HCC recurrence, the issue of how specific immunosuppressive drugs affect the disease process is poorly understood.[35] Obviously, a regimen that can perform its immunosuppressive function that is necessary for preventing graft rejection but concomitantly exerts antitumor effects should be the preferential clinical choice in this particular setting. In this aspect, mTOR inhibitors attract attention. However, only approximately 50% of all HCC patients exhibit activation of mTOR downstream signaling elements in their tumors.[10,36] Indeed, both experimental and clinical evidence suggest that tumors bearing different genetic mutations can respond differentially to mTOR inhibitors.[37,38] Given the heterogeneity of HCC, other immunosuppressive regimens also deserve careful attention. Several studies have reported that MPA could inhibit cancer cell proliferation across different types of cancer cell lines (Table S2, SDC, http://links.lww.com/TP/B692) as well as potentially supportive evidence from patients (Table S3, SDC, http://links.lww.com/TP/B692).

In this study, we have demonstrated an anticancer effect of MPA in experimental HCC models, including human HCC cell lines and mouse primary liver tumor organoids. Culture of primary liver cancer cells from either human or mouse has been proven to be very difficult. The organoid technology (culturing "mini-organ" in three dimension) has endowed the possibility of establishing stable cultures from primary tumors, including for liver tumors.[27,28,34] Our data support that MPA has potent inhibitory effects on HCC growth in vitro. More importantly, clear inhibition of mouse liver tumor organoid initiation and growth was also observed after MPA treatment. We further provided clinical evidence that the use of MMF, the prodrug that metabolizes into MPA after administration, is associated with reduced disease recurrence and improved survival in

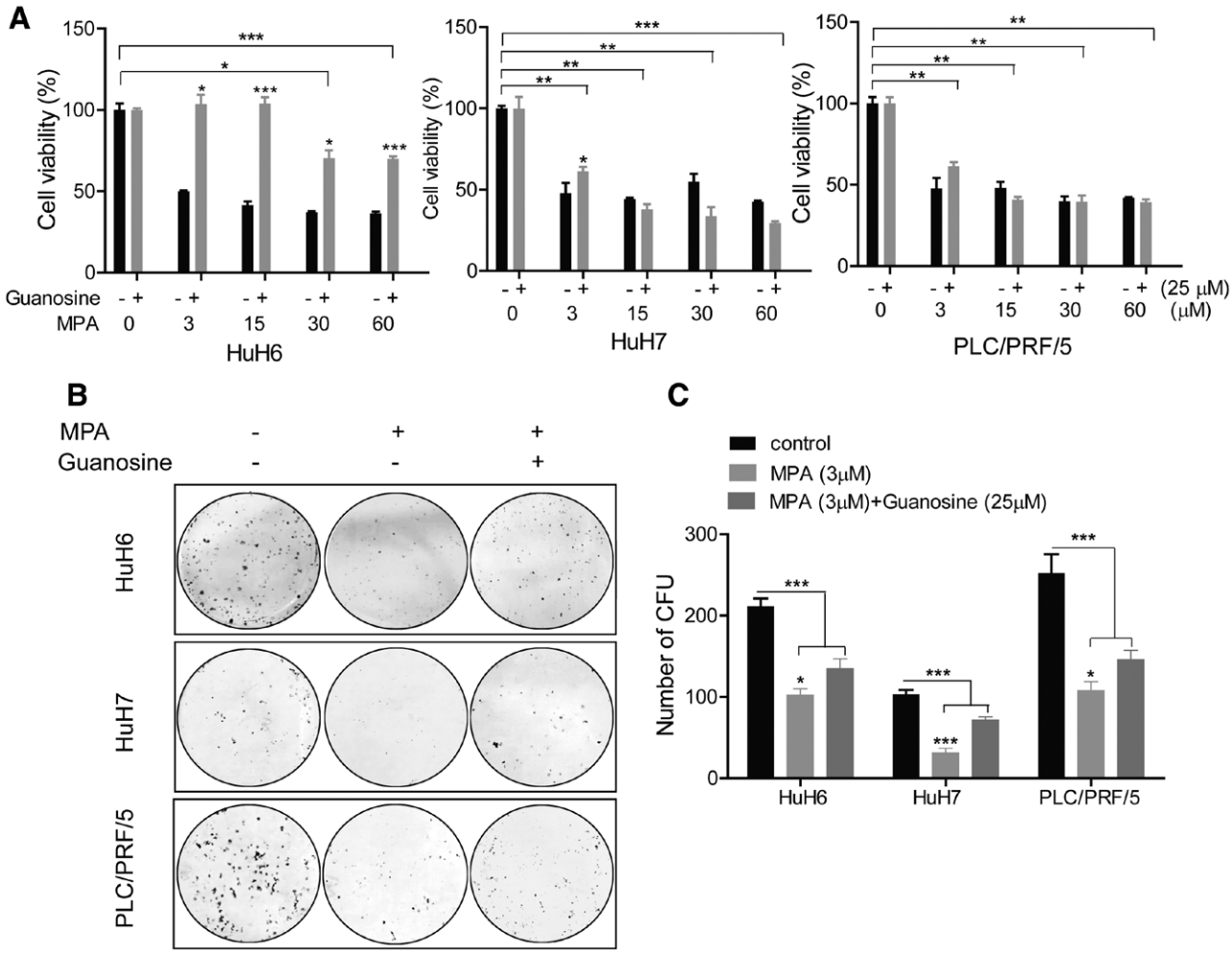


FIGURE 5. Guanosine supplementation partially counteracts effects of MPA. MTT assay of HuH7, HuH6, and PLC/PFR/5 cell lines (A) and CFU assay of HuH6 and PLC/PRF/5 cell lines treated with MPA and/or guanosine (B) showed that exogenous guanosine could partially counteracted the effect of MPA; (C) quantification of CFU assay (mean ± SEM, n = 6, $^{***}P < 0.005$). Results from at least 3 independent experiments are shown. CFU, colony-forming unit; MPA, mycophenolic acid; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; SEM, standard error of the mean.

HCC-related LT patients. These results indicated an antitumor action of MPA occurring.

Although the antitumor effects of MPA have been substantially established, it is still unclear how this drug exerts the antitumor activity. Several molecular pathways appear to play a pivotal role in MPA-induced apoptosis.[39] Two p53-induced genes (*TP53I3* and *TP53INP1*), as well as the p53 protein, are known to be upregulated by MPA.[39] The increase of p53 level provides a mechanism for rapid growth arrest or apoptosis in the event of DNA damage during S phase of cell cycle.[40] In our study, the induction of S phase arrest in HCC cells by MPA is in agreement with these known findings. We surprisingly found that supplementation of exogenous guanosine counteracts only to a minor extent to the inhibitory effect of MPA in HCC cells. Although depletion of guanine nucleotide pools is the predominate mechanism in inhibiting lymphocyte proliferation, this however only partially explains the mechanism of action in anti-HCC by MPA. The responsiveness to guanosine supplementation was different among the 3 different HCC cell lines, which might be due to the distinct intrinsic features of these cell lines. Of note, different from the response to sorafenib, even high concentration of MPA cannot completely eradicate all the cancer cells. There are several possible explanations. First of all, there is a salvage pathway to synthesize nucleotides from intermediates in the degradative pathway that may counteract the inhibitory effect of MPA. Interestingly, our previous study has demonstrated that fast-cycling cancer cells are sensitive, but slow-cycling cancer cells are resistant to MPA treatment.[41]

Excitingly, after performing a retrospective analysis in our LT cohort, we found an association between MMF use and reduced HCC recurrence and improved patient survival. Importantly, there are no significant differences regarding patient and tumor characteristics[29] between these 2 groups. It must be said that our observations may also be related to a potential inferior immunosuppressive effect of MMF containing treatment regimens. Because of the small sample size, single-center setting, and retrospective nature of these findings, further clinical evaluation is warranted preferentially in randomized studies to confirm our findings. Moreover, 3 of 23 other IMPDH inhibitors were found to possess both stronger immunosuppressive and antitumor activity than MPA

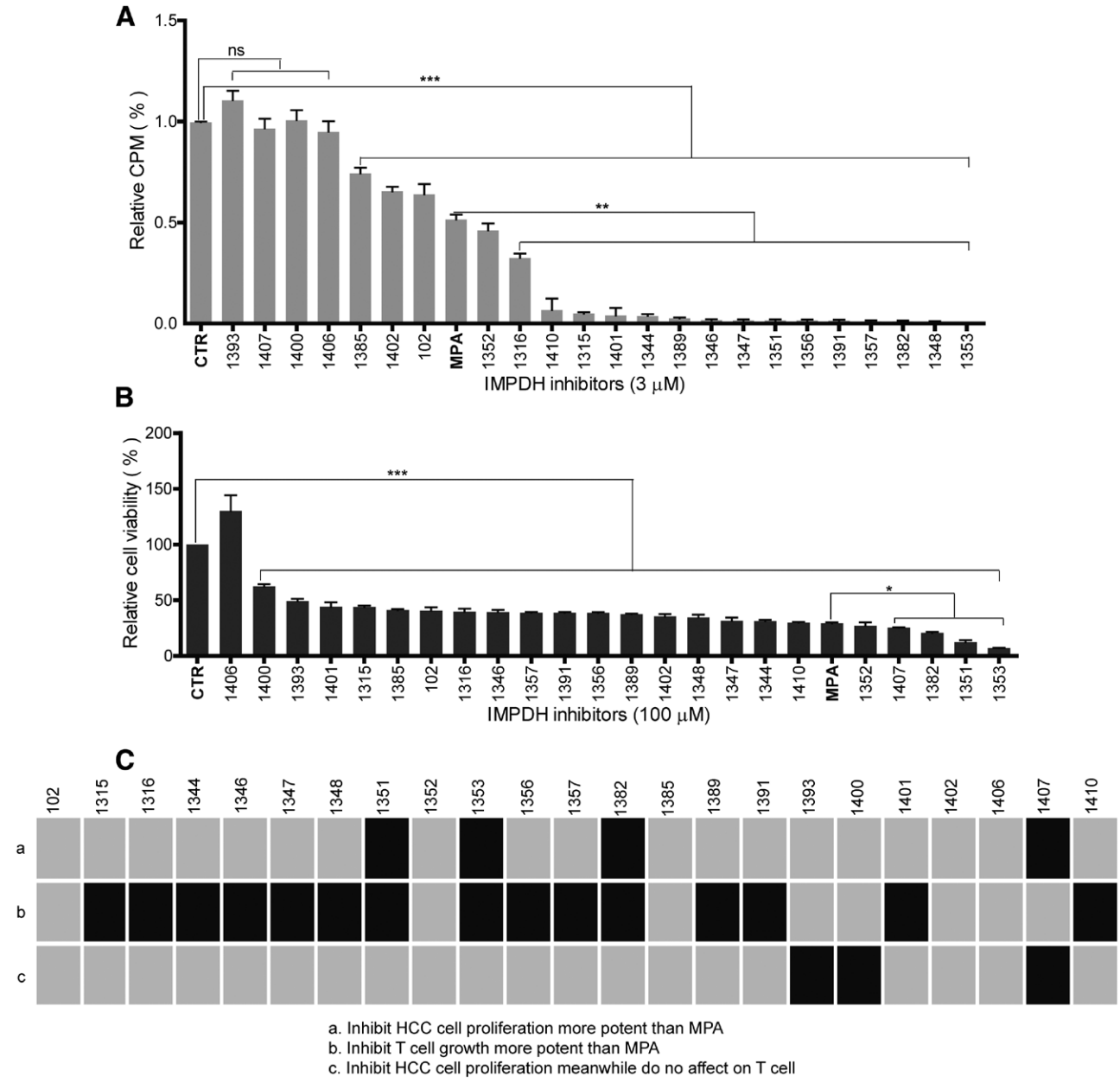


FIGURE 6. Other IMPDH inhibitors and their immunosuppressive and anti-HCC activity. A, [3H]-Thymidine assay showed that 15 compounds were more potent than MPA in inhibiting T-cell proliferation (mean ± SEM, n = 3, $^{**}P < 0.01$). B, MTT assay showed that 4 compounds were more potent than MPA in inhibiting HuH6 cells proliferation (mean ± SEM, n = 3, $^{*}P < 0.05$). C, Three compounds were verified to be more potent in inhibiting T cells and HuH6 cells than MPA (mean ± SEM, n = 3, $^{**}P < 0.01$). Three compounds could inhibit HuH6 cells proliferation without effecting T-cell proliferation (compounds vs CTR, mean ± SEM, n = 3, $^{***}P < 0.001$). Results from at least 3 independent experiments are shown. CPM, counts per minute; CTR, control; HCC, hepatocellular carcinoma; IMPDH, inosine monophosphate dehydrogenase; MPA, mycophenolic acid; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; SEM, standard error of the mean.

and may therefore be considered as potential alternatives for MMF in the LT set.

In summary, this study has demonstrated that clinically relevant concentrations of MPA are capable of constraining HCC cell growth in experimental models. We further provided clinical evidence that MMF is associated with reduced HCC recurrence and improved survival in LT patients. Confirming these experimental findings and retrospective clinical observations by prospective randomized trials could lead to better management of immunosuppressive medication for HCC patients after LT.

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