Identification of Type 1 Inosine Monophosphate Dehydrogenase as an Antiangiogenic Drug Target

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Abstract: To rapidly discover clinically useful angiogenesis inhibitors, we created and screened a library of existing drugs for inhibition of endothelial cell proliferation. Mycophenolic acid (MPA), an immunosuppressive drug, was found to potently inhibit endothelial cell proliferation *in vitro* and block tumor-induced angiogenesis *in vivo*. Using RNA interference, we found that knockdown of one of the two known isoforms of inosine monophosphate dehydrogenase (IMPDH-1) is sufficient to cause endothelial cell cycle arrest.

Drug discovery is time-consuming and expensive; on average it takes 14.8 years and $800 million to bring a new drug to the clinic.[1] One way to accelerate this process involves finding new uses for existing drugs. Because the toxicity, pharmacokinetics, and clinical properties of existing drugs are well established, compounds that show activity can be rapidly and inexpensively evaluated as new treatments and moved into the clinic if appropriate. Furthermore, the extensive structure−activity data accumulated during the development of each drug can greatly facilitate mechanistic studies including target identification or validation. Although several groups have reported unexpected new properties of established drugs that make them useful in treating new diseases,[2−4] only recently has a systematic approach been taken to discovering new applications for existing drugs using partial libraries of the estimated 10 000 pharmaceuticals known to date.[5−7] Partial libraries of FDA-approved drugs have been screened for the ability to induce differentiation of acute myeloid leukemia cells,[6] protect against glutamate neurotoxicity,[7] work in combination to offer novel antifungal and antitumor activity,[8] and block polyglutamine protein aggregation.[9] The success of this approach prompted us to initiate an effort to systematically collect and screen all known drugs for novel pharmacological activities.

We created a library of 1850 FDA-approved drugs and 600 drugs that entered the clinic via approval in a foreign country or phase II clinical trials (for more details, check www.jhccsi.org). To discover new angiogenesis inhibitors among the known drugs, we screened the library for inhibition of human umbilical vein endothelial cells (HUVEC) using [^{3}H]thymidine incorporation. An initial screen of the drugs at 10 μM (final concentration) revealed over 100 drugs that exhibited at least 50% inhibition. After eliminating known cytotoxic drugs such as paclitaxel and colchicine or drugs that are restricted to topical use, several drugs remained. One of the most potent inhibitors was mycophenolic acid (MPA) (Figure 1A). The IC_{50} for MPA on HUVEC proliferation was 99.2 ± 5.2 nM (Figure 1B). Although MPA has been previously shown to inhibit endothelial cells,[10,11] the molecular basis of this inhibition remains unclear. Nor has it been shown whether MPA affected angiogenesis *in vivo*. We thus initiated a series of experiments to address these questions.

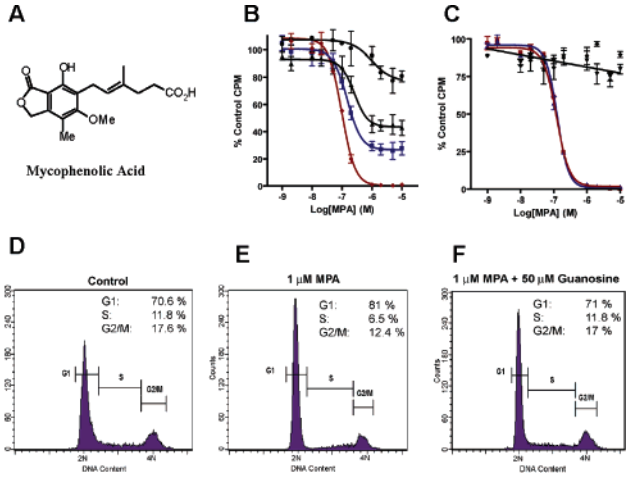

Figure 1. Inhibition of HUVEC by MPA and its reversal by guanine: (A) structure of mycophenolic acid; (B) inhibition of HUVEC proliferation by MPA as measured by [^{3}H]thymidine incorporation. MPA inhibition (◆) is partially reversed by addition of exogenous guanine at 10 μM (■) and 20 μM (▲); however, some inhibition is present even at the highest concentrations tested, 50 μM (●). (C) Inhibition of Jurkat T cell proliferation by MPA as measured by [^{3}H]thymidine incorporation. MPA inhibition (◆) is unaffected by addition of 10 μM exogenous guanine (■) but is completely reversed by the addition of 20 μM (▼) and 50 μM (◆) guanine, in contrast to HUVEC. (D) Cell cycle analysis of HUVEC treated with DMSO vehicle, 1 μM MPA showing G1/S cell cycle arrest (E), or 1 μM MPA + 50 μM guanosine (F). Addition of exogenous guanosine reverses MPA-induced G1/S cell cycle arrest.

MPA is an immunosuppressive drug widely used to prevent rejection of transplanted organs.[12] The mechanism of action in the immune system is well established; MPA inhibits the *de novo* guanine nucleotide biosynthesis.[13] Because the alternative nucleotide salvage pathway is absent in T and B cells, MPA was thought to specifically inhibit their proliferation by causing cell cycle arrest in the G1/S transition.[14] We thus compared HUVEC to Jurkat T cells for sensitivity to MPA in the presence and absence of guanine. As shown in Figure 1C, Jurkat T cells are inhibited by MPA with an IC_{50} of 128 ± 6.1 nM, which is comparable to IC_{50} values previously reported in human peripheral blood T and B lymphocytes.[14] Addition of guanine at or above 20 μM rendered Jurkat T cells resistant to MPA. Similar to Jurkat T cells, inhibition of HUVEC proliferation is also reversed by guanine in a dose-dependent manner (Figure 1B). Unlike Jurkat T cells, however, HUVECs are less sensitive to guanine, the cause of which remains unknown. Similar

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reversal of MPA inhibition is also seen in HUVEC and Jurkat T cells with the addition of exogenous guanosine and deoxyguanosine (data not shown).

It has been shown that MPA causes cell cycle arrest in activated T and B lymphocytes at G1.[15] We thus examined the effect of MPA on endothelial cell cycle progression. Treatment of HUVEC with 1 μM MPA led to, in comparison to the control cells, an increase in the population of cells in the G1 phase (81% vs 71%) and a corresponding decrease in the population of cells in S (6.5% vs 12%) and G2/M (12.4% vs 17.8%) phases, indicating that MPA also causes a G1 blockade in HUVEC similar to T and B cells (Figure 1D,E). Addition of 50 μM guanosine completely reversed the cell cycle effect of MPA in HUVEC (Figure 1F). Together, these results strongly suggest that blockade of purine biosynthesis is responsible for the inhibition of HUVEC by MPA, similar to T and B cells.

The molecular target for MPA in T and B cells has been unambiguously established as inosine monophosphate dehydrogenase (IMPDH), which catalyzes the NAD^+-dependent conversion of inosine 5′-monophosphate to xanthosine 5′-monophosphate.[13] Two isoforms of IMPDH are known in humans: type 1 and type 2 enzymes.[16] We assessed expression of the two isoforms in actively proliferating HUVEC using RT-PCR. It was found that IMPDH-1 is the predominantly expressed isoform in HUVEC, although IMPDH-2 mRNA is also detected (data not shown). This expression pattern is similar to that in peripheral leukocytes but distinct from those in most other tissues in which IMPDH-2 is more abundantly expressed.[17] To determine whether inhibition of either isoform of IMPDH accounts for the effect of MPA on HUVEC, each isoform was knocked down by RNA interference. To avoid subjecting the primary HUVEC to the relatively harsh conditions of conventional transfection methods, lentiviruses were used to deliver isoform-specific shRNAs to HUVEC.[18,19] When three different regions of each IMPDH cDNA were tested, at least one shRNA construct was found that efficiently blocked the expression of IMPDH-1 or IMPDH-2 with high specificity (Figure 2A). Transduction of HUVEC with a mixture of lentiviruses carrying shRNAs for both IMPDH-1 and IMPDH-2 led to the knockdown of mRNA (Figure 2A) and protein (data not shown) for both isoforms. The effects of knockdown of the two isoforms of IMPDH on the cell cycle of HUVEC were then determined. As shown in Figure 2B,C, knockdown of IMPDH-1 is sufficient to cause a cell cycle arrest in G1. Interestingly, knockdown of IMPDH-2 appeared to cause a significant delay in the S phase progression rather than a G1 blockade (Figure 2D). Not surprisingly, knockdown of both isoforms of IMPDH also led to accumulation of HUVEC in G1 (Figure 2E). These observations validated IMPDH-1 as the target for MPA in endothelial cells.

To test the *in vivo* efficacy of MPA as an angiogenesis inhibitor, we performed the Matrigel plug angiogenesis assay in mice using doses previously used to demonstrate immunosuppression in murine transplant models.[20,21] Matrigel plugs containing VEGF and bFGF were subcutaneously implanted into mice. The control group was treated with saline vehicle, and the MPA group was treated with 60 or 120 (mg/kg)/day of drug. After 10 days, plugs from control mice showed extensive neovascularization that is visible macroscopically (Figure 3A) and microscopically (Figure 3C). In contrast, MPA treated mice had significantly less new blood vessel formation (Figure 3B,D). To quantify these differences, we counted erythrocyte-filled blood vessels per 100× field (Figure 3E)[21] and observed a 69% decrease in new blood vessel formation in mice treated with

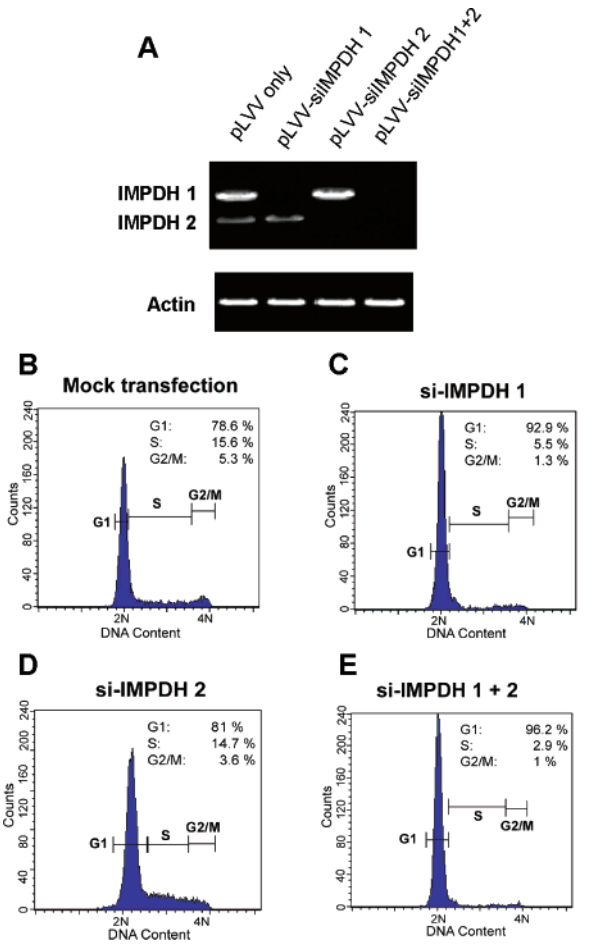

Figure 2. Selective knockdown of IMPDH-1 and -2 by shRNA in HUVEC. (A) RT-PCR of IMPDH-1 and -2 knockdown in HUVEC. The IMPDH-1 and -2 shRNA vectors completely and selectively abolish IMPDH gene expression in HUVEC. (B) Cell cycle analysis of HUVEC transfected with empty vector. sh-IMPDH-1 (C), sh-IMPDH-2 (D), and sh-IMPDH-1 + -2 (E) show a G1/S arrest similar to that observed with MPA treatment. This G1/S arrest is more pronounced with the shIMPDH-1 construct.

MPA in comparison with vehicle control ($p < 0.002$). Thus, MPA is capable of inhibiting angiogenesis at a therapeutically achievable dose *in vivo*.

Angiogenesis has been implicated in tumor growth among a number of other diseases.[22] Although MPA inhibits fast-growing tumor cell lines in culture and in mouse xenograft experiments,[23,24] it is unclear whether MPA also affects tumor-associated angiogenesis. We determined the efficacy of MPA in inhibiting tumor-associated angiogenesis in a murine renal cell carcinoma (RENCA) model.[25] MPA inhibited the growth of the primary tumor in a dose-dependent fashion, causing a 34% and 27% decrease in volume and weight, respectively, at 60 (mg/kg)/day and a 64% decrease in volume and weight at 120 (mg/kg)/day ($p < 0.001$) (Figure 4A). The decrease in tumor growth caused by MPA at 120 (mg/kg)/day was accompanied by a 48% decrease in the area of CD31 positive staining blood vessels per 200× field ($p < 0.001$). As shown in Figure 4B,C, whereas CD31 positive blood vessels were abundant in tumors from control animals, MPA treatment at 120 (mg/kg)/day led to a significant reduction in CD31 positive vessels in the primary tumor. These results demonstrate that MPA is capable of decreasing tumor-induced angiogenesis *in vivo*.

The selectivity of MPA for T and B lymphocytes was thought to be due to dependence on the *de novo* nucleotide synthesis pathway for proliferation. Endothelial cells, along with smooth

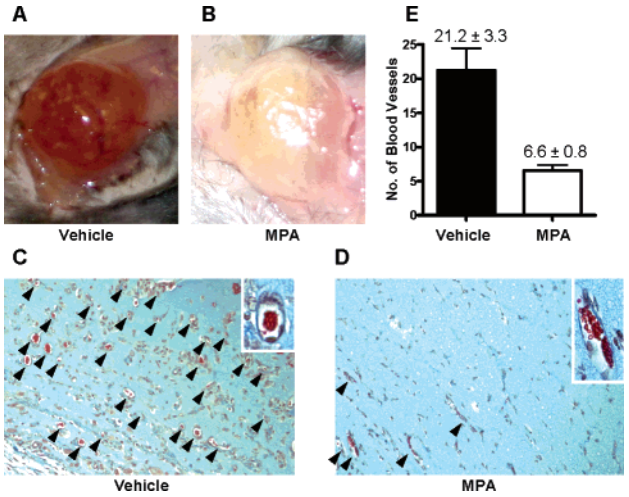

Figure 3. MPA inhibits angiogenesis *in vivo*: representative images of Matrigel plugs in mice treated with vehicle ($n = 7$) (A) or 60 (mg/kg)/day MPA ($n = 7$) (B). MAS-trichrome stain of Matrigel plug from a mouse treated with vehicle demonstrating new blood vessel formation (100× magnification, 200× inset) (C) or MPA (60 (mg/kg)/day sc) (D) and showing a 69% decrease in new blood vessel formation ($p < 0.002$) (E).

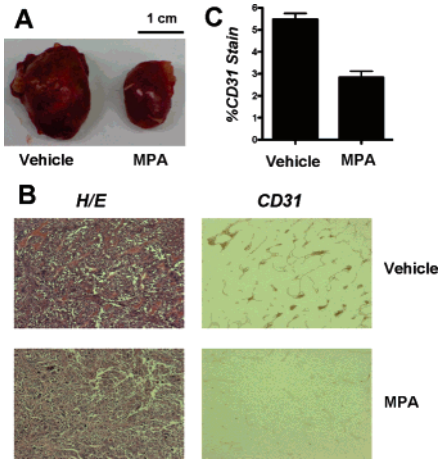

Figure 4. MPA inhibits tumor-associated angiogenesis: (A) representative primary RENCA tumors from mice treated with vehicle ($n = 7$), left, or 120 (mg/kg)/day MPA ($n = 8$), right, showing decrease in tumor size. (B) H/E cross section (100×) of representative RENCA tumor from control mice shows extensive angiogenesis, with blood vessels highlighted by CD31 staining. In contrast, representative tumor sections from mice treated with 120 (mg/kg)/day MPA stained with H/E and CD31 show decreased angiogenesis compared to control. (C) Quantification of CD31 positive area per 200× microscope field shows a 49% decrease in mice treated with 120 (mg/kg)/day MPA ($p < 0.001$).

muscle cells, fibroblasts, and epithelial cells, were thought to be less sensitive to blockade of *de novo* nucleotide synthesis because inhibition of proliferation is seen at relatively high concentrations of MPA compared to T and B cells.[11,14] The reduced sensitivity of these cell types to MPA was attributed to the use of the nucleotide salvage pathway. Here, we demonstrate the unequivocal role of *de novo* nucleotide synthesis in endothelial cell proliferation. MPA is as potent at inhibiting the proliferation of HUVEC as Jurkat T cells (Figure 1B vs 1C) as well as primary T and B cells as reported previously.[14] The underlying cause for the difference in sensitivity of endothelial cells to MPA between this study and previous reports remains unknown. Unlike T cells, we found that much higher concentrations of guanosine are required to completely reverse inhibition in HUVEC (Figure 1B), indicating that *de novo* nucleotide synthesis plays a more essential role in endothelial cell proliferation than in T and B cells. This difference in dependence on *de novo* nucleotide synthesis between endothelial and T cells raised the possibility that angiogenesis may be more susceptible to inhibition by MPA *in vivo*.

MPA and its prodrug form, mycophenolate mofetil, have been in clinical use for a number of years as an immunosuppressant. Its peak plasma level in human renal transplant patients undergoing chronic oral treatment is 75 μM with a half-life of 18 h, which is nearly 750-fold higher than the IC_{50} for inhibition in endothelial cells.[26] MPA has been shown to inhibit the growth of tumor cells *in vitro* and in mouse xenografts.[24] This observation led to testing of MPA in small cohorts of patients (<35) with a variety of cancers in the 1970s.[27,28] Because the diethanolamine salt of MPA was used rather than the currently prescribed mofetil prodrug, considerable dose-limiting gastrointestinal toxicity occurred.[27] Given our current understanding of angiogenesis, MPA would be expected to slow the rate of tumor progression, which was not the endpoint used in previous studies in cancer patients.[27,28]

Although the inhibitory effects of MPA on endothelial cells have been observed previously, it was not clear whether the same molecular mechanism underlies the antiangiogenic and the immunosuppressive effects. In this study, we establish for the first time that the antiangiogenic activity of MPA in endothelial cells shares the same molecular basis as its immunosuppressive effects in T and B cells. More importantly, we validate IMPDH as the target for MPA in endothelial cells using RNA interference delivered through the noninvasive lentivirus transduction of primary endothelial cells. With isoform-specific siRNA constructs, we found that knockdown of IMPDH-1 is sufficient to cause the same cell cycle effect in endothelial cells as MPA treatment. The physiological functions of both isoforms of IMPDH have been investigated using gene knockout in mice. Of the two isoforms, IMPDH-2 appears to be more essential because the homozygous knockout of murine IMPDH-2 led to early embryonic lethality.[29] In contrast, IMPDH-1 null animals developed normally and exhibited no obvious defects.[30] Importantly, the function of T cells from proliferation to cytokine production was largely intact in IMPDH-1 knockout mice, suggesting that this isoform is dispensable for T cell development and function. Indeed, when the two IMPDH isoforms were knocked down individually using siRNA in Jurkat T cells, it was found that IMPDH-2, rather than IMPDH-1, is essential for the proliferation of Jurkat T cells (see Supporting Information). Together, these findings raise the exciting possibility that isoform-specific inhibitors of IMPDH-1 may be selective for endothelial cells without affecting the immune system and devoid of the side effects of MPA and other existing nonselective IMPDH inhibitors.

This work is based on the premise that there exist unappreciated physiological activities among known clinical drugs. This premise was proven by the identification of multiple known drugs with unexpected inhibitory effects on endothelial cell proliferation *in vitro* and angiogenesis *in vivo*. In addition to the endothelial cell proliferation assay, we have screened the library in a number of other cellular assays. We find that the hit rates with this drug library are significantly higher than commercially available small-molecule libraries on more than half a dozen cellular screens. The molecular basis of these high hit rates may lie in the shared genome and largely overlapping proteome of all human cell types and tissues. Significant redundancy exists in the usage of individual genes in different physiological and pathological processes. Thus, there is great

potential in screening existing drugs for novel biological and therapeutic activities.

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Supporting Information Available: Figure S1 of isoform-specific knockdown of IMPDH-1 and -2 in Jurkat T cells. This material is available free of charge via the Internet at http://pubs.acs.org..

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