TM5484 ameliorates collagen IV deposition in CNS of EAE mice, it could be also related to its anti-inflammatory effects. For instance, van Horssen and colleagues showed that excessive deposition of collagen IV in CNS of patients with MS might be caused by increased production of cytokines [34]. TM5484 did not affect on the amount of fibrin deposition in spinal cord, suggesting that PAI-1-enhanced by EAE might affect on the infiltration of inflammatory cells to a higher degree than extra cellular matrix components.

There are a number of ways in which PAI-1 may contribute to the development of inflammation and demyelination in the CNS. For instance, PAI-1 can act as a chemoattractant for macrophages [10]. In the present study, gene expression and histological analyses consistently indicated that TM5484 treatment attenuated macrophage migration and microglia activation into the spinal cord, thereby attenuating demyelination and axonal damage.

Neurotrophic factors are known to protect neurons against various pathological insults. For instance, BDNF is a growth factor that plays important roles in the development and maintenance of the nervous system and induces neuronal survival [35]. In this study, EAE mice exhibited decreased mRNA expression of BDNF compared to control animals, while TM5484 treatment its BDNF expression. Inhibition of PAI-1 is predictably associated with a reciprocal increase in the activity of its target serine protease, t-PA, with secondary increases in net plasmin generation. Both of these serine proteases are known to convert secreted proBDNF to mature BDNF in the synaptic cleft [14]. In addition to its effects on demyelination and axonal degeneration, TM5484 may also accelerate neuronal repair in diseases linked to decreased BDNF levels. Although we only found differences of BDNF expression at the gene level, these findings suggest that TM5484, directly or indirectly modulate BDNF expression in neurons and thus might provide an additional mechanism for neuroprotection. To reinforce this, TM5484 also up-regulated the expression of ChAT, meaning a reduction of neuronal loss in spinal cord of EAE mice. This suggests that the effect of PAI-1 inhibitor TM5484 goes beyond blockage of inflammation, as it also induces prevention of damage and preservation of neuronal tissue. In contrast to previous studies using a mice model of Rett syndrome [36], our data showed that fingolimod did not restore the expression of mRNA BDNF in EAE mice.

Finally, the observed benefits of TM5484 were extended experimentally in rats. Admitting that the paralytic behavior seen in EAE rats is usually induced by infiltrating cells as well as by edema caused by blood-brain barrier disruption rather than by demyelination [37], when TM5484 was initiated at the time of onset of signs, motor paralysis was reduced to levels observed in untreated control, indicating that TM5484 also benefits the rat model of MS.

The mechanistic understanding of MS has advanced considerably over the past decade and has provided the rationale for the application of anti-inflammatory and immunomodulatory treatments that can actually reduce the severity and frequency of new demyelinating episodes [38]. Since TM5484 appears to influence several important pathogenic mechanisms in MS, it merit further investigation and consideration as a novel therapeutic modality for the treatment of MS.

In conclusion, we report evidence in this study that inhibition of PAI-1 with a low-molecular compound protect mice against EAE-induced inflammation, demyelination and axonal degeneration. TM5484 represents thus a novel therapeutic approach for MS and, perhaps, other CNS disorders.

Supporting Information

S1 Fig. Flow-chart of the identification of PAI-1 inhibitors efficiently penetrating thru the BBB. Based on virtual screening and *in silico* docking simulation we first developed a new group of oral, low molecular PAI-1 inhibitors. Among over 500 derivatives of the lead

compound TM5275 we searched for a PAI-1 inhibitor that could efficiently penetrate into BBB. About 50 compounds, which met the criteria for a good CNS penetration, *i.e.*, a low MW with a lipophilicity (clogP) < 4 and a surface area (TPSA) > 75, were selected and tested for CNS penetration, using an *in vitro* model that corresponds to the anatomical situation of cerebral microvessels. The penetration ratio (Papp) was finally measured. TM5484 (M.W., 384.7; clogP, 3.07; TPSA, 108.6), a derivative of TM5441 (Boe *et al* 2013), exhibited the highest penetration ratio across the BBB (Papp of 67.6 x 10^{-6} cm/s). It was selected for subsequent experiments.
(EPS)

S2 Fig. Gene expression. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of Tnf-1, IL-1b, IL-6, IL-10, IL-17 and IFNγ in spleen. EAE mice show an increased expression of proinflammatory cytokines as well as a decreased expression of IL-10. TM5484 and fingolimod were able to modulate these inflammatory effects. Expression levels of all markers are normalized to b-actin. Data are shown as the means and corresponding SEM. $^{**}P<0.01$ by 1-way ANOVA and Dunnett test, n = 6–7
(EPS)

S3 Fig. Gene expression. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of ChAT in spinal cord. TM5484 as well as Fingolimod up-regulated ChAT expression comparing to EAE untreated mice. Expression levels of all markers are normalized to b-actin. Data are shown as the means and corresponding SEM. $^{**}P<0.01$ by 1-way ANOVA and Dunnett test, n = 5–6
(EPS)

S4 Fig. In spinal cord of control mice, expression of Iba-1 marker shows low levels of microglia activation, as demonstrated by none ramified branches. EAE mice show an important number of microglia activated with ramified branches (green). However, this was ameliorated by TM5484 and fingolimod. Nuclear Dapi staining (blue) confirmed the presence of viable cells.
(PDF)

S5 Fig. Immunostaining. Fibrinogen deposition in spinal cord of EAE mice shows no difference in comparison to control. In addition, no changes were observed after treatment with TM5484 or fingolimod. Red asterix indicates central canal.
(PDF)

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Author Contributions

Conceived and designed the experiments: NP TD AI TM. Performed the experiments: NP TD AI TM HS. Analyzed the data: NP TD AI TM DV CY. Wrote the paper: NP TD AI TM DV CY.

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