L'acido micofenolico in reumatologia: meccanismi d'azione ed eventi avversi gravi*

Mycophenolic acid in Rheumatology: mechanisms of action and severe adverse events

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RIASSUNTO

L'acido micofenolico (MPA) è un agente immunosoppressore, sempre più utilizzato in campo trapiantologico, reumatologico e nefrologico. In questa rassegna, analizzeremo i meccanismi d'azione del farmaco, apportando le più recenti acquisizioni, che suggeriscono nuove applicazioni terapeutiche del MPA, come la fibrosi, il danno vascolare e l'ipertensione polmonare, in corso di connettivite.

La somministrazione di adeguate dosi giornaliere e la terapia basata sul monitoraggio del farmaco potrebbero essere determinanti per garantire in vivo le concentrazioni di MPA necessarie a spegnere l'infiammazione e ripristinare la tolleranza.

Occorrerà, tuttavia, tenere conto dei possibili eventi avversi gravi (i.e. leucoencefalopatia multifocale progressiva [PML]) legati all'immunosoppressione.

INTRODUCTION

MPA is clinically administered as morfolino-ethyl ester (mycophenolate mofetil, MMF) or, more recently, as a salt (enteric-coated mycophenolate sodium).

It is a fermentation product of *Penicillium brevicompactum* and other analogue fungi, identified by Gosio in 1893 as a weak antibacterial agent; in 1969 Franklin and Cook discovered its capacity to inhibit the inosine monophosphate dehydrogenase (IMPDH), an enzyme involved in purine nucleotide synthesis (1).

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In particular, IMPDH converts inosine monophosphate, produced from adenosine monophosphate by adenosine deaminase (ADA), in guanosine monophosphate.

By increasing the guanine nucleotide pool, ADA and, subsequently, IMPDH, exert a positive feedback on *de novo* nucleotide synthesis; in fact, 5-phosphoribosyl-1-pyrophosphate synthetase and ribonucleotide reductase are finally stimulated (2). Allison et al. (2), observing that children with ADA deficit were severely immunodeficient, grasped the strategic role of IMPDH in lymphocytic development and proliferation, and tested MPA as an immunosuppressive agent.

Lymphocytes constitute the main target of MPA, because they require *de novo* nucleotide synthesis, and also because they specifically express the type II IMPDH isoform (IMPDH-2), which is 5-fold more potently inhibited by MPA compared to the ubiquitarian type I isoform (2).

However, MPA affects many other cell types; moreover, some effects seem to be IMPDH-independent, since not reversible in presence of exogenous guanosine *in vitro* (Tab. I).

Effects on lymphocytes

Proliferation

At low concentration, MPA (100 nM) inhibits T and B lymphocyte proliferation in response to mitogens *in vitro*, and suppresses mixed leukocyte reactions (MLR) even when incubated 3 days after, indicating it acts by blocking DNA synthesis (3).

In fact, MPA specifically inhibits the late activation surface markers (CD154, CD71) (4). Additionally, MPA blocks IL-2 and IL-15-induced proliferation, since it impedes IL-2-mediated down-regulation of the cycline (cyc)-dependent kinase (CDK) inhibitor p27/Kip1 (4); thus, CycD/CDK6 and CycE/CDK2 complex activation fails and cells cannot transit from G1 to S phase.

Table I - Mechanisms of action of MPA.

Effect	Cellular targets	Molecular mechanisms (observed or hypothesized)	IMPDH-dependent (GTP depletion)	References
Proliferation inhibition	Lymphocytes, monocytes, endothelial cells, fibroblasts, mesangial cells, VSMC	DNA synthesis inhibition; CDK inhibitor p27/Kip1 maintenance	Yes ?	(3; 5; 21; 40-41; 48), (4)
Apoptosis induction	Lymphocytes, monocytes	Caspase induction	?	(5-7)
Necrosis induction	Lymphocytes	RhoGDI-2 cleavage by caspase-3 leads to Cdc42 up-regulation and cytoskeleton changes	?	(8-9)
Cytokine production inhibition	T lymphocytes, monocytes, DCs, endothelial cells	mRNA synthesis inhibition; NF-kB inhibition	Yes ? (No)	(6; 10-11; 15; 21) (20-21)
Immunoglobulin production inhibition	B lymphocytes	mRNA synthesis inhibition (?)	? (Yes)	(12-14)
TLR-induced cell activation inhibition	B lymphocytes, DCs	MD-1 protein reduction; p38MAPK inhibition; NF-kB inhibition (?)	? No ?	(10) (31; 39)
Surface protein expression and cell-cell interaction inhibition	Monocytes, DCs, endothelial cells	mRNA synthesis inhibition; membrane protein N-glycosylation inhibition; p38MAPK inhibition	Yes Yes No	(17; 19; 21; 27-30) (18) (31)
ECM synthesis inhibition and cytoskeleton alterations	Fibroblasts, mesangial cells, VSMC	mRNA synthesis inhibition; Rac1 inhibition and b-calp up-regulation; PDGF-B and Egr-1 down-regulation	Yes Yes No	(39-43) (45; 48) (47)
Oxidative stress inhibition and vascular protection	Endothelial cells, neutrophils, mesangial cells, VSMC	Rac1 and PKC inhibition leads to Nox inhibition; Lack of tetrahydrobiopterin leads to iNOS inhibition; ET-1 decrease and PGI2 increase	Yes Yes ?	(25; 33; 48) (24) (22-23)
Degranulation inhibition	Mast cells	G-protein inhibition and cytoskeleton dysfunction (?)	? (Yes)	(50)

Apoptosis
MPA induces apoptosis in lymphocytes and monocytes (5-6), including superantigen (staphylococcal enterotoxin B)-activated T lymphocytes (7); that would be important in transplantology, since the interaction with MHC-II by superantigens is analogue to that by alloantigens (*direct allorecognition*) and is critical in acute organ rejection.

Necrosis
MPA also causes lymphocytic necrosis *in vitro*, through an atypical mechanism dependent on G-protein Cdc42 recruitment and subsequent actin polymerization; indeed, necrosis would be preponderant, earlier and would require lower MPA concentrations compared to apoptosis (8).
Cdc42 up-regulation might follow regulatory protein RhoGDI-2 (Rho GTP dissociation inhibitor-2) cleavage mediated by caspase-3 (9).
Thus, MPA-induced caspase activation might lead to cell death via both apoptosis and necrosis.

Cell function
MPA inhibits cytokine production (TNFα, IFNγ, IL-12, IL-10, IL-4) by T lymphocytes in animal models and SLE patients (6, 10); additionally, MPA (1 μM) blocks IL-15-induced IL-17 production by peripheral blood-derived T cells, even more strongly than cyclosporine A and leflunomide metabolite A77-1726 (11). MPA inhibits immunoglobulin production by B cells and their differentiation in memory cells and plasma cells (12, 13).
In SLE patients, it diminishes the circulating autoantibody levels (14). Moreover, MPA may interfere with Toll-like receptor (TLR)-mediated activation of B lymphocytes and dendritic cells (DCs), via MD-1 protein reduction (10).

Effects on monocytes
MPA (10 μM) is antiproliferative and proapoptotic on monocytes and monocyte precursors (3, 5), induces the terminal differentiation of monocytes in macrophages (5), reduces IL-1β and increases IL-1 receptor antagonist production (15).
Furthermore, MPA reduces monocyte and lymphocyte chemotaxis to inflammation sites (16), via ICAM-1 and MHC-II expression inhibition (17) and adhesion protein N-glycosylation blockade, the latter due to intracellular guanosine nucleotide depletion and, subsequently, guanosine triphosphate (GTP)-dependent fucose and mannose membrane transfer impairment (18).

Effects on endothelial cells
MPA (5-20 μM) inhibits the cytokine-induced expression of E-selectin (CD62E), VCAM-1 (CD106), CD34, ICAM-1 (CD54), NF-kB and IL-6 production (19-21).
It impedes endothelial migration, proliferation and angiogenesis *in vitro* (21).
In addition, MPA reduces endothelin-1 (ET-1) expression (22) and increases prostacyclin (PGI$_2$) release (23); it suppresses the cytokine-induced production of nitric oxide (NO) (24), presumably because of lack of GTP-derived tetrahydrobiopterin, essential coenzyme of the inducible NO synthetase (iNOS).
Moreover, MPA (1-10 μM) inhibits superoxide anion production by endothelial NADPH-oxidase (Nox), since GTP depletion leads to the inactivation of Rac1, a G-protein involved in Nox activity (25). Instead, the effects on ICAM-1 and IL-6 seem to be IMPDH-independent (21).

Effects on dendritic cells
Similarly to what previously observed in murine models of delayed contact hypersensitivity (26), also in human monocyte-derived DCs, MPA (10 μM) reduces the surface expression of costimulation and interaction molecules (CD40, CD54, CD86, CD80, CD83) and cytokine production (TNFα, IL-12, IL-18) (27).
Moreover, TNFα-stimulated DCs incubated with MPA (100 μM) do not acquire maturation morphologic features and continue to express immature cell receptors, like CXCR1 (28).
In addition, a recent study on myeloid DCs found that MPA (100 μM) increases the expression of chemokine receptor CCR1 and decreases CCR7 levels, and strongly contrasts the effects of TLR3 ligation on DC activation and maturation, which include the down-regulation of receptors for inflammatory cytokines (CCR1, CCR2, CCR5) and the up-regulation of lymph node chemokine receptors (CCR7); basically, MPA, inhibiting DC homing from periphery to lymph nodes, would interfere with key events in breaking peripheral tolerance in AID or in provoking a chronic allograft rejection in transplant recipients (29).
Other authors found that MPA 50 μg/mL (corresponding to 150 μM) also leads to CD205 down-regulation, probably contributing to antigen uptake impairment (30). LPS-stimulated and MPA-pretreated DCs show decreased phosphorylation of p38 MAPK (mitogen-activated protein kinase), necessary for DC maturation (31).

Allogeneic T cells incubated with MPA-treated DCs are not only impeded to proliferate (28, 29), but also acquire suppressive activity against control T cell proliferation; in fact, they become antigen-specific and contact-dependent regulatory T cells (Treg), expressing high levels of CD25, Foxp3, CTLA-4, CD95 (Fas), and producing high amounts of IL-10 and TGFβ (32).

The block of DC maturation and tolerogenicity could be due to both IMPDH-dependent and IMPDH-independent mechanisms: guanosine depletion may provoke surface marker mRNA synthesis impairment (30); on the other hand, inhibition of both p38 MAPK phosphorylation in DCs and allogeneic T cell proliferation in co-cultures is not reversible after guanosine addition (28, 31).

The great majority of studies on DCs typically considered much higher MPA concentrations (more often 100 μM) compared to experiments on other cell types (more often up to 10 μM); in fact, only 100 μM may guarantee the full down-regulation of costimulatory and adhesion markers in DCs and the induction of a specific chemokine receptor expression pattern (29).

Effects on neutrophils

Whereas initial observations seemed to exclude MPA effects on neutrophils, MPA was subsequently found to suppress TNFα-induced endothelial adhesion of neutrophils (21). MPA (1-10 μM) also inhibits superoxide anion and hydrogen peroxide production in neutrophils activated *in vitro*, because of Nox activation impairment by Rac1 or Protein kinase C (PKC) (25, 33).

Nevertheless, an increase in hydrogen peroxide production was even observed after 30 minutes from neutrophil activation; that might explain the paradoxical acute inflammatory syndrome rarely reported in patients treated with MMF (34-35).

Effects on fibroblasts

MMF therapy suppresses TGFβ expression in human transplanted kidney biopsies (36), similarly to what initially observed in a rat renal transplant model (37). MMF treatment also leads to TGFβ level reduction in the lungs of lupus-prone MRL/lpr mice (38).

Additionally, MMF (0.1-10 μM) decreases type I collagen and increases MMP-1 expression, and inhibits α-smooth muscle actin expression, hallmark of the myofibroblast phenotype (39).

MPA (10 μM) almost abolishes fibroblast proliferation and significantly down-regulates gene expression of several cytoskeletal proteins; actin and tubulin filaments lose their regular orientation and vinculin does not localize in focal adhesions, with subsequent decrease in FAK (focal adhesion kinase) phosphorylation and gross alterations of fibroblasts, which acquire an ovoid shape and lose migration, adhesion and wound-healing skills (40).

Effects on mesangial cells

MPA (1-10 μM) inhibits proliferation and extracellular matrix (ECM: type I collagen and fibronectin) production in human mesangial cells, and their contraction and migration skills *in vitro* (41). Recently, MPA was found to inhibit anti-DNA antibody-induced PKC activation and subsequent TGFβ and fibronectin synthesis in human mesangial cells, and reduced proteinuria in lupus-prone mice (42).

MPA also suppresses fibronectin and oxygen radical production by murine mesangial cells stimulated by high-dose glucose (43), and MMF can prevent nephrin and podocin loss in experimental diabetic nephropathy, with a marked attenuation of proteinuria (44).

Rac-1 inactivation, as a consequence of MPA-induced GTP depletion, leads to an increase of basic calponin (b-calp), a protein associated to actin fibers; that would finally cause mesangial cell inactivity (45) and would confer protection in glomerulonephritis (46).

Furthermore, MPA down-regulates PDGF-B and PDGF-BB induced Egr-1 (early growth response gene-1) expression in rat mesangial cells; these effects are not reversible after guanosine addition (47).

Table II - Rheumatic diseases treated with MPA.

Rheumatic disease	References
Lupus nephritis	(14; 53-57)
Lupus non-renal manifestations	(reviewed in 58)
ANCA-related vasculitis	(59-62)
Systemic sclerosis	(63-65)
Idiopathic inflammatory myopathies	(reviewed in 52)
Takayasu arteritis	(66)
Behçet disease	(67)
Sjögren syndrome	(68)
Rheumatoid arthritis	(reviewed in 69)
Psoriatic arthritis	(70)

Effects on vascular smooth muscle cells

MPA (0.1-10 μM) inhibits rat vascular smooth muscle cell (VSMC) proliferation, their production of collagen, fibronectin and oxygen radicals, and Rac-1 activity (48).

In the rat monocrotaline model of pulmonary hypertension, MMF significantly reduces the medial thickness of pulmonary arteries and the right ventricle wall thickness, macrophage infiltration and the endothelial expression of P-selectin and IL-6 (49). MPA also inhibits the proliferation of VSMCs derived from human pulmonary arteries *in vitro* (49).

Effects on mast cells

MPA (1-10 μM) inhibits rat mast cell degranulation, leading to a significant decrease in serotonin release (50). That may explain MMF efficacy in the treatment of refractory chronic idiopathic urticaria (51).

Effects on rheumatic patients

MMF has been successfully used in several rheumatic diseases (Tab. II) (14, 52-70).

Thanks to its efficacy against kidney transplant rejection and its nephroprotective properties, studies mainly focused on lupus nephritis (LN) as both induction (53-57) and maintenance (14) therapy, and on ANCA-positive vasculitides (59-62).

Moreover, the antifibrotic role of MPA, added to its actions in favor of endothelial protection and against experimental pulmonary hypertension, encouraged studies on systemic sclerosis (SSc) (63-65). The better results in chronicity index at re-biopsy in LN patients on MMF compared to those on cyclophosphamyde (CTX) (55), and the lower occurrence of clinically significant pulmonary fibrosis in MMF-treated SSc patients compared to control groups (64), may be at least partly consequent to MPA antifibrotic action.

Critical issues concerning treatment outcomes are the daily dose administrated and the drug concentration achieved *in vivo*.

Low MMF-dosing could have been responsible for the high rate of relapse, mainly experienced in vasculitis patients (60-62); also in LN management a slower rate of MMF tapering was soon adopted (54). Whereas MMF 2 g/day was found to be comparable to CTX in LN induction therapy (53-55), MMF 3 g/day seemed to be even superior to CTX (56).

A subsequent larger trial did not confirm such a result, even though asserted the non-inferiority of MMF compared to CTX; nevertheless, MMF was found superior to CTX in Black and Hispanic patient group (57).

It is noteworthy that, although the target daily MMF dose in this study was 3 g/day, the mean dose actually given was 2.47 g/day; moreover, the median dose was higher in Black and Hispanic patients (2.8 g/day) than in White and Asian patients (2.6 g/day) (57).

To date, MMF dose of 2 g/day is considered comparable to azathioprine as LN maintenance therapy (14), whereas 3 g/day is recommended as LN induction therapy.

The great majority of studies on other rheumatic diseases tested MMF at 2 g/day or less, and 3 g/day have been only recently considered in SSc patients (65); therefore, better results are expected from next studies on full-dose therapy.

On the other hand, there is an increasing interest on therapeutic drug monitoring of MPA in non-transplanted patients, particularly AID patients (71-73). In fact, low plasma albumin levels, proteinuria >1 g/24 h, renal failure (74-75), concomitant medications (cyclosporine (74), steroids (76), proton-pump inhibitors (77), etc.), and genetics (enzyme polymorphisms (78)) can heavily interfere with MPA exposure, and may explain the great inter-individual variability observed in MPA pharmacokinetics.

Thus, a fixed-dose MMF therapy could not guarantee the concentrations required for MPA effects on cells *in vivo*.

In AID (Lupus and vasculitis) patients taking MMF 2 g/day for at least 10 weeks prior to the study, clinical endpoints significantly correlated with MPA trough levels at 12 h (pre-dose levels, C_{12h}), but not with MMF dose (72); in particular, the disease relapsed in 41% of patients with C_{12h} <2 mg/L, in 29% of those with C_{12h} <3 mg/L, in 2% of those with C_{12h} 3-3.5 mg/L and in none of the patients with C_{12h} ≥3.5 mg/L.

These authors suggested a target range for C_{12h} of 3.5-4.5 mg/L (quantified by high-performance liquid chromatography), as an upper threshold of 4.5 mg/L best discriminated between patients with and without adverse events (72).

In AID patients, C_{12h} is significantly correlated to MPA-area under the concentration *versus* time curve (AUC) at 12 h (71, 72).

In Lupus patients treated with MMF for 31±30 months at a mean dose of 1.6±0.5 g/day, other authors found a significant correlation between C_{12h} and C4 complement fraction levels: specifically,

patients with C4 consumption or normal C4 concentrations had mean C_{12h} values equal to 1.7 or 3.8 mg/L, respectively (quantified by enzyme-multiplied immunotechnique) (73). Thus, MPA trough levels ≥3-3.5 mg/L may be adequate for maintenance of remission.

These levels correspond to MPA concentrations proved to be sufficient for *in vitro* effects on numerous cell types (10 μM, equal to 3.2 mg/L). Instead, many MPA actions on DCs would require about 10-fold higher concentrations; therefore, it remains questionable to what extent DCs are actually affected by MMF treatment *in vivo*.

In this regard, not MPA trough levels but rather MPA maximum concentrations (C_{max}) might predict the achievement in the patient of a congruous dose of MPA (virtually ≥32 mg/L) capable of not only switching off inflammation but also restoring peripheral tolerance.

It is known that both C_{max} and AUC rapidly increase in the first three months of therapy, whereas rise more slowly later (79). In fact, a subset of AID patients taking MMF 2 g/day for at least 10 weeks reached C_{max} values ≥32 mg/L (mean C_{max} 21.8±14.09 mg/L) (71).

Severe adverse events
At any rate, the risk of severe adverse events associated to immune suppression must be minded during MPA dosing assessment.

Actually, MMF therapy has been related to several infections, sometimes life-threatening, by viruses like cytomegalovirus, poliomavirus, varicella (80, 81).

The infection risk would be higher compared to that of patients on azathioprine (80, 82). Critical factors seem to be daily dose, leucopenia, virus-specific IgM level decrease (82, 83).

Importantly, MMF use may be associated to PML, interpreted as an opportunistic infection of the brain by JC and BK poliomavirus (84, 85).

In a large retrospective cohort study of renal transplant recipients, the incidence density of PML in MMF users was 14.4 cases/100,000 person-years at risk versus 0 for non-MMF users, although the difference was not statistically significant; pretransplant transfusion and use of antirejection medications in the first year were found to be favoring factors (85).

CONCLUSION

MPA seems to represent a precious tool in the hands of rheumatologists, since it has pleiotropic effects on both immune and non-immune cells, resulting in immune suppression, fibrosis inhibition, renal and vascular protection.

Further studies are needed to assess whether full-dose therapy and therapeutic drug monitoring can confer additional advantages in the management of AID patients.

SUMMARY

Mycophenolic acid (MPA) is an immunosuppressive agent, more and more extensively used in transplantation, rheumatology and nephrology.

In this review, we will analyze the molecular mechanisms of its action, including the newest insights, in particular the inhibition of lymphocytes and the induction of tolerogenic dendritic cells (DCs) and its direct effects on non-immune cells (fibroblasts and myofibroblasts, mesangial cells, vascular smooth muscle cells [VSMC], endothelial cells).

The latters suggest new therapeutic indications, specifically fibrosis (i.e. glomerulosclerosis and interstitial lung diseases), vascular damage and pulmonary hypertension, which represent key pathogenic features in connective tissue diseases.

Given the differences in sensitivity to MPA among the various cell types and the great inter-individual variability in MPA pharmacokinetics, adequate daily doses and therapeutic drug monitoring may be decisive to ensure those MPA concentrations needed to switch off inflammation and restore peripheral tolerance in autoimmune disease (AID) patients.

A warning on the severe adverse events strictly linked to immune suppression (i.e. progressive multifocal leukoencephalopathy [PML]) will be stressed.

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