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LETTER TO THE EDITOR
(in memory of Professor Enrico Triolo)

Antiviral activity of mycophenolic acid derivatives in plants

A. PANATTONI[1], A. LUVISI[1*], S. FUSELLI[1], F. D.ANDREA[2], F. GIORGELLI[2], L. GUAZZELLI[2], G. CATELANI[2], E. TRIOLO[1]

[1]Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto 80, 56124 Pisa, Italy; [2]Department of Pharmacy, University of Pisa, Via Bonanno Pisano 6, 56126 Pisa, Italy

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Mycophenolic acid (MPA) is a potent inhibitor of inosine monophosphate dehydrogenase (IMPDH), a key enzyme in the *de novo* synthesis of guanine nucleotide (*1*). MPA exhibits anti-proliferation activity and has been established as an anti-cancer agent, an immunosuppressant (*1, 2*) as well as an anti-viral agent against plant viruses (*3, 4, 5, 6, 7, 8*). Significant efforts were made on MPA structural modifications and the MPA derivatives elicited interesting biological properties with increased antiviral activity (*9, 10*). These studies confirmed the significance of the MPA core structure as potential pharmacophore. A recent and interesting way to display pharmacophores is to load them on dendrimers. These structures often show multivalent effects which make them attractive scaffolds for drug delivery systems (*11*). Dendrimers are nanostructured macromolecules characterized by a tree-like architecture with exponential numbers of discrete dendritic branches radiating out from a common core with each branch exposing a unit of bioactive drug (*12*). Polyamidoamine (PAMAM) dendrimers represent the first class of dendrimers to be characterized and extensively investigated (*13*).

*Corresponding author. E-mail: aluvisi@agr.unipi.it; phone: +39 0502210560.

Abbreviations: MPA = mycophenolic acid; IMPDH = inosine monophosphate dehydrogenase; PAMAM = polyamidoamine; IMP = inosine monophosphate; XMP = xanthosine monophosphate; ME = multivalency effect

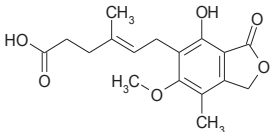
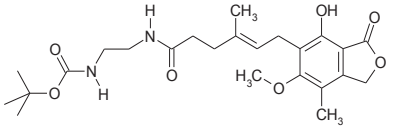
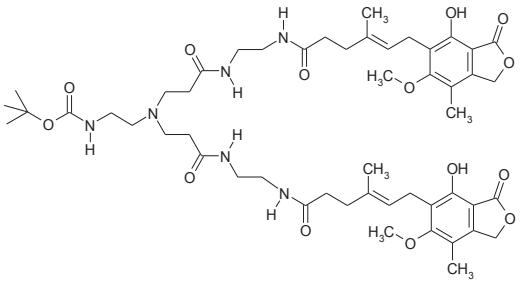
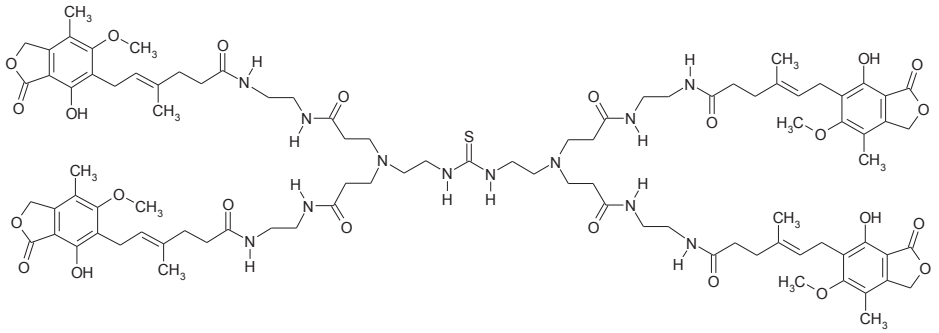
The aim of this study is to evaluate toxicity and antiviral activity of MPA molecules loaded onto PAMAM dendrimers. Tests were carried out in *in vitro* plant experimental systems (*Nicotiana tabacum* L. cv. Xanthi infected by cucumber mosaic virus) which represent a rapid way to assay drugs antiviral activity in few weeks (*14*).

A series of MPA derivatives - Amide A, Dendrimer A, and Dendrimer B - were synthesized (Table 1). All compounds were characterized by ^{1}H and ^{13}C NMR and elemental analysis (data not shown), confirming chemical structure and purity of MPA derivatives.

Drugs toxicity was evaluated in *in vitro N. tabacum* L. cv. Xanthi explants. *In vitro* tobacco explants were obtained following D'Anna (*14*) protocols. Toxicology studies involved administration of the separate drugs, to six consecutive subcultures incubated for 15 days, compared to control (no drug). Healthy tobacco explants were treated with MPA and the synthesized derivatives at several concentrations (0.00, 0.10, 0.20, 0.30, and 0.40 mmol/l). The threshold of toxicity, expressed as mortality rate, was set at 10% of dead explants.

Antiviral activity was expressed as percentage of virus-negative explants (% of virus-negative explants = virus-negative explants at the end of treatments/virus-positive explants at the beginning of treatments). The screening of virus-negative explants was carried out by DAS-ELISA (*15*) after each subculture (15 days long, repeated six times) and by RT-PCR (*5*) after the last subculture. Drugs dosage was set at 0.30 mmol/l (*14*). Tissue samples from healthy (HC)

Table 1. Chemical structure of MPA and its derivatives

MPA and derivatives	Chemical structure
MPA	
Amide A	
Dendrimer A	
Dendrimer B	

and infected (IC) explants were used as negative and positive controls, respectively. For DAS-ELISA tests, readings were normalized as R values (OD-treated explant/OD-HC); R = 2.0 was used as threshold to distinguish a positive response vs. a negative response (*16*). For RT-PCR tests, carried out on DAS-ELISA-negative explants, fragments were amplified with specific primers (*17*). In order to evaluate the multivalency effect (ME) of derivatives, antiviral activity of derivatives ($AA_{derivatives}$) was compared to antiviral activity of MPA (AA_{MPA}). ME was calculated using the equation:

$$ME = (AA_{derivatives}/AA_{MPA} \times 100) - 100$$

All the experiments were performed in triplicate; each experiment consisted of 15 explants infected with CMV virus. The effects of treatments were determined using analysis of variance (ANOVA).

No toxic effects were observed on tobacco healthy explants after administration of MPA and synthesized structures up to 0.30 mmol/l (Table 2). At higher dosage, toxicity exceeds the threshold for MPA and derivatives. The non-toxic dosage used for antiviral activity trials was 0.30 mmol/l for all compounds tested. Toxicity was dosage- and time-dependent and not drug-dependent (data not shown). In the literature there are two examples of low toxicity of MPA in plants, according to data reported by D´Anna (*14*) on tobacco and Skiada *et al.* (*18*) on *Vitis vinifera* and its PAMAM dendrimers studied in this work show the same behavior.

With regard to DAS-ELISA-negative explants, MPA effectiveness was lost for Dendrimer B and reduced for Amide

Table 2. Toxicity expressed as mortality (%) of healthy *Nicotiana tabacum* L. cv. Xanthi explants at the end of subculturing (90 days) treated with drugs at different dosage and antiviral activity of MPA derivates ($AA_{derivatives}$) and MPA (AA_{MPA}) against cucumber mosaic virus and multivalence effectiveness of derivatives [$ME = (AA_{derivatives}/AA_{MPA} \times 100) - 100$]

Compounds	Drug dosage (mmol/l)	Mortality (%)	ELISA-negative (%)	Antiviral activity (%)	ME (%)
MPA	0.30	4.4	24.3	13.3	0.0
	0.40	17.7			
Amide A	0.30	6.7	13.2	4.4	-76.9
	0.40	15.5			
Dendrimer A	0.30	4.4	7.9	0.0	-100.0
	0.40	15.5			
Dendrimer B	0.30	4.4	0.0	0.0	-100.0
	0.40	17.7			

A and Dendrimer A (Table 2). This trend was confirmed for the antiviral activity which was retained only by Amide A. However, Amide A was 76.9% less effective than MPA as antiviral compound. Loss of antiviral activity by Dendrimer A and Dendrimer B indicated that these PAMAM dendrimers did not show any ME.

In medical research, attempts to obtain analogues of MPA with improved properties advances the elucidation of the structure-activity relationship of MPA in its interaction with IMPDH and contributes to the understanding of the relevant sites required for drug activity: the interaction of the aromatic phtalide nucleus and hypoxanthine nucleus, the network of H-bonding of C-phenol and lactone with Thr 333, Gln 441 and Gly 326, the ionic and H-bonding interactions of the carboxylic acid side chain with Ser 276 (*9, 19*). To better understand the activity of MPA as inhibitors on IMPDH it is useful to refer to the kinetic mechanism of the IMPDH catalyzed biochemical conversion of IMP to XMP. This occurs by initial nucleophilic attack of an active site of cysteine (Cys 331) at the 2-position of IMP to form a covalent intermediate (E-IMP*). Subsequent hydride transfer to the nicotinamide ring of the cofactor, NAD^+, followed by hydration of the resulting intermediate results in the formation of the tetrahedral intermediate, E-XMP. The final step is the expulsion of XMP from the latter intermediate (*19*). MPA binds to the enzyme after the ternary complex is formed and inhibits IMPDH activity by trapping the covalent intermediate preventing the hydrolytic attack at the C-2 position and blocking the proceeding of reaction (*20*). Even though the MPA derivatives were designed in light of these literature evidences, our findings showed that the retention of these parameters does not guarantee the increase – or at least the maintenance – of antiviral activity. In our tests, the toxicity of MPA derivatives was maintained – as dosage – and time-dependent – suggesting a similar drug-cell interaction. The low antiviral activity of MPA when single-loaded on scaffold in Amide A suggests that maintaining the structural performances of bioactive sites of MPA is not enough to obtain potentiated drug derivatives and further research is needed.

Instead, the loss of antiviral activity of Dendrimer A and Dendrimer B seems to be related to the scaffold. It is well known that for the branched dendritic architecture subtle, and yet important, parameters are able to control the interior space of a dendrimer and to influence guest–host interactions. These include crucial branch-cell components, such as: branching angles, rotation angles, and repeat-unit segment length. Of equal importance are the spatial, physicochemical and multiplicity properties of the core (*13*).

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