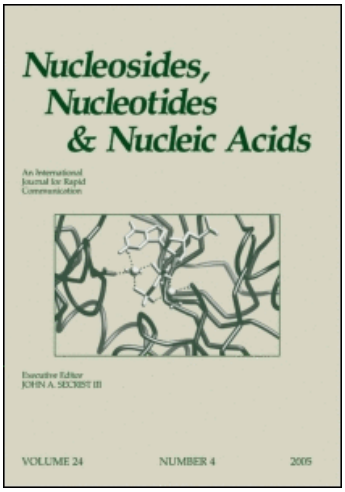

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Potential Role of Mycophenolate Mofetil in the Management of Neuroblastoma Patients

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ABSTRACT

In human neuroblastoma cell lines (LAN5, SHEP and IMR32), mycophenolic acid (MPA) at concentrations (10^{-7}–10^{-6} M) readily attainable during immunosuppressive therapy with mycophenolate mofetil (Cellcept), induces guanine nucleotide depletion leading to cell cycle arrest and apoptosis through a p53 mediated pathway (up-regulation of p53, p21 and bax and down-regulation of bcl-2 and survivin). MPA-induced apoptosis is also associated to a marked decrease of p27 protein. In the same cell lines MPA, at lower concentrations (50 nM), corresponding to the plasma levels of the active free drug during Cellcept therapy, induces differentiation toward the neuronal phenotype by causing a partial chronic guanine nucleotide depletion. MPA-induced differentiation is not associated to p27 accumulation as occurs using retinoic acid. At a fixed concentration of MPA a higher percentage of apoptotic or differentiated cells is obtained when non dialysed serum substitutes for the dialysed one, due to the higher hypoxanthine concentration in the former (about 10 μM) leading to competition on HPRT-mediated salvage of guanine. At hypoxanthine or oxypurinol concentrations higher than 1 μM (up to 100 μM) no further enhancement of MPA effects was obtained, in agreement with the recently described safety of the allopurinol-mycophenolate mofetil combination in the treatment of hyperuricemia of kidney transplant recipients. The apoptotic effects of MPA

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do not appear to be significantly increased by the UDP-glucuronosyltransferase inhibitor niflumic acid.

Key Words: cGMP; CAMP; PDEs inhibitors; Neuroblastoma proliferation and differentiation.

INTRODUCTION

Mycophenolic acid (MPA) specifically inhibits inosine-5′-monophosphate dehydrogenase (IMPDH; EC 1.1.1.205), the enzyme catalyzing the rate-limiting reaction for the de novo synthesis of guanine nucleotides. Although MPA demonstrated potent cytotoxicity in vitro, it did not exhibit this activity in vivo, probably due to extensive mycophenolate glucuronide formation.[1] However, when administered as its ester pro-drug form, mycophenolate mofetil (Cellcept, Roche, Basel, Switzerland), MPA exerted cytostatic effects on lymphocytes and nowadays is one of the most commonly employed immunosuppressive agents.[1] Furthermore this morpholinoethyl ester pro-drug showed a marked in vivo antitumor activity in athymic nude mice bearing some human tumor xenografts.[2]

We have investigated the in vitro effects of MPA, at concentrations readily attainable during immunosuppressive therapy, on three human neuroblastoma cell lines (LAN5, SHEP and IMR32).

MATERIALS AND METHODS

Exponentially growing cells were treated with MPA (ranging from 0 to 10 μM) in the presence or in absence of hypoxanthine or niflumic acid (up to 100 μM), 10 μM guanine or 100 μM guanosine for 0 to 6 days. Cell cycle phases were measured by flow cytometry. Measurement of cell survival was performed using the cell proliferation reagent WST-1. Apoptosis was measured by flow cytometry using a two color TUNEL assay. Nucleotides in protein free extracts were analyzed by RF-HPLC. Cellular localization of p53 protein was studied by immunocytochemistry. p53 and p27 protein expression was determined by Western blot analysis. RT-PCR was used to analyze mRNA levels of p53, p21, bax, bcl-2, survivin and p27. Cell differentiation was assessed by morphological changes and by immunohistochemical detection of differentiation markers (MAP-5, NF-200, GAP-43, Laminin and Fibronectin).

RESULTS AND DISCUSSION

Mycophenolic acid (0.1–10 μM) caused a decrease of intracellular levels of guanine nucleotides, a G1 arrest and a time- and dose-dependent death by apoptosis. After 48 hour treatment with 1 μM MPA intracellular GTP and GDP concentrations dropped to 49% and 64% of control cells (incubated with no added drug), respectively. After 72 h of incubation in the presence of 1 μM MPA, 65% of LAN5, 56% of IMR32 and 28% of SHEP were apoptotic while in the absence of the drug the percentage of apoptotic cells was 3%, 13% and 1% respectively.

G1 cell cycle arrest and apoptosis appear to be induced by MPA through a p53 mediated pathway. RT-PCR and western blot analyses have shown that after MPA treatment the amount of p53 mRNA and protein expression were increased with a shuttling of p53 protein into the nucleus. According to the hypothesis that p53 inhibits IMPDH[3] it could be suggested that MPA activates an amplification loop for IMPDH inhibition and p53 induction. Evidence that MPA treatment leads to activation of p53 dependent pathways stems from RT-PCR results, demonstrating up-regulation of p21 and bax and down-regulation of bcl-2 and survivin in MPA treated cells. MPA induces also a time- and dose-dependent decrease of p27 protein expression evidenced by western blot but not by RT-PCR analysis in agreement with the observation that p27 level is mainly regulated by the rate of its degradation.[4]

MPA-induced effects (G1 arrest, apoptosis, activation of p53-dependent pathways, down regulation of p27 protein expression) are triggered by guanine nucleotide depletion. They are reversed by addition of guanosine (0.1 mM) together with the IMPDH inhibitor to the culture medium, at least partially restoring GTP and GDP levels through their salvage. Furthermore, at a fixed concentration of MPA, a higher percentage of apoptotic cells is obtained when non dialysed serum substitutes for the dialysed one, perhaps due to the higher hypoxanthine concentration in the former leading to competition on HPRT-mediated salvage of guanine.[5]

To try to enhance MPA-induced effects, hypoxanthine plasma levels can be increased by the administration of allopurinol. Safety of the allopurinol-mycophenolate mofetil combination in the treatment of hyperuricemia of kidney transplant recipients has been reported.[6] At concentrations up to 100 μM and with an incubation time of 3 days, hypoxanthine and oxypurinol are not cytotoxic for the neuroblastoma cell lines employed. However, as shown in the figure, a rise of hypoxanthine concentration above the mean plasma levels found in healthy subjects (about 1 μM) does not significantly enhance the apoptotic effects of MPA.

Mycophenolic acid (MPA) is primarily metabolized to its 7-O-glucuronide by at least three UDP glucuronosyltransferase forms (UGT1A8, UGT1A10 and UGT1A9).[7] Niflumic acid has been reported to inhibit MPA glucuronidation.[8] Niflumic acid was not cytotoxic when neuroblastoma cells were incubated for three days in the presence of the drug at concentrations (100 μM) corresponding to the highest plasma levels obtained after a standard 350 mg oral dose. At these concentrations only a slight enhancement of the MPA-induced apoptotic effects was observed (Fig. 1).

Mycophenolate mofetil is rapidly absorbed following oral administration and rapidly and almost completely hydrolyzed to the active metabolite MPA which is extensively and tightly bound to human albumin.[9] Free MPA is the pharmacologically active fraction and in most patients accounts for about 2% of the mean plasma drug level which is usually maintained around 3 μg/ml.[9] MPA, at concentrations of the active free drug readily attainable therapeutically using mycophenolate mofetil, induces differentiation toward the neuronal phenotype in all human neuroblastoma cell lines studied (LAN5, IMR32 and SKNSH).

After six days of incubation in the presence of the drug (50 nM) extension of neurite like processes were well evident in LAN5 and in SKNSH. Although these morphological changes were less marked in IMR32 cells, in all neuroblastoma cell lines an up-regulation of some neuronal differentiation markers was demonstrated by immunohistochemical analysis. In cells treated with 50 nM MPA there was a slight reduction of guanine nucleotide pools (RF-HPLC), an activation of p53 dependent pathways with an

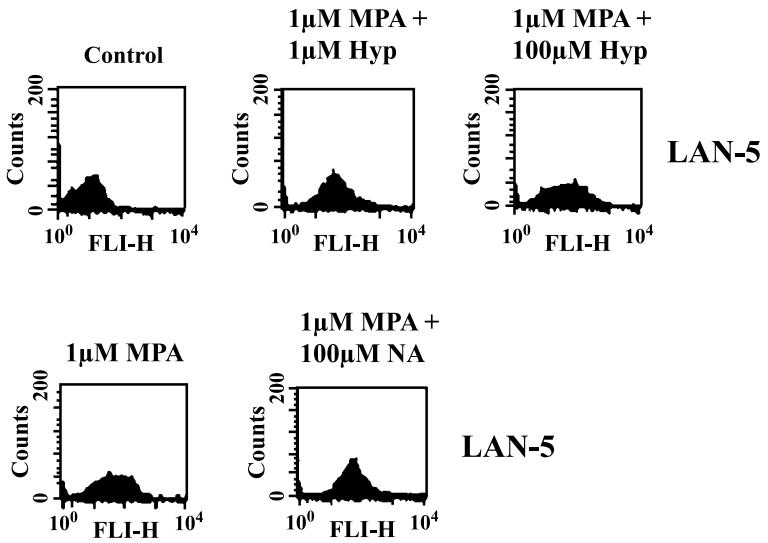

Figure. 1 Effects of hypoxanthine (Hyp.) and of niflumic acid (NA) on mycophenolic acid (MPA)-induced apoptosis in LAN5 cells incubated at 37°C for 72 h with 0 μM (Control, 5% apoptosis), 1 μM MPA (60% apoptosis) or 1 μM MPA in the presence of 1 or 100 μM Hyp (57% apoptosis) or 100 μM NA (68% apoptosis). Apoptosis was quantified by flow cytometry using a two color TUNEL assay. Histograms of increased green fluorescence intensity, respect to that of controls correspond to increased apoptosis.

up-regulation of p53, p21 and bax and a down-regulation of bcl2 and survivin (RT-PCR). p27 protein levels were comparable to those of control cells or only slightly decreased (western blot). All these effects were, at least partially, reversed by the addition of 0.1 mM guanosine together with the IMPDH inhibitor to the culture medium. The molecular mechanisms involved in MPA-induced differentiation appear to be different from those triggered by retinoic acid involving a decrease in the levels of p53 and p53 mRNA[10] and an accumulation of p27 protein.[11]

In conclusion Cellcept appears to have a potential role in the management of neuroblastoma patients. In vitro MPA plasma levels reached at the doses employed in immunosuppressive regimen lead neuroblastoma cells to die by apoptosis, while concentrations corresponding to the free levels of the drug induce neuroblastoma cells to differentiate toward the neuronal phenotype. At least in vitro the apoptotic effects of the drug do not appear to be significantly increased by the UDP-glucuronosyltransferase inhibitor niflumic acid. The in vitro obtained data lead to suggest that the combination of allopurinol with Cellcept could not be useful to enhance the apoptotic effects of the drug on neuroblastoma cells.

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