

ORIGINAL**The nucleoside and nucleotide mixture (OG-VI) rescues intestinal-like epithelial cells from the cytotoxicity of chemotherapeutic agents**

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Abstract : Immune cells and cells undergoing rapid turn-over can obtain exogenous nucleotides via salvage synthesis. We evaluated whether or not the balanced nucleoside and nucleotide mixture OG-VI, could rescue intestinal epithelial-like Caco-2 cells from the cytotoxic effects of several chemotherapeutic agents, in the presence and absence of glutamine (Gln). Cells were exposed to 5-fluorouracil (5FU), methotrexate (MTX) or 6-mercaptopurine (6MP), after which proliferation and cell cycle analyses were performed. Following exposure to the chemotherapeutic agents, we observed that cells treated with OG-VI proliferated well, whereas those without the supplement did not proliferate. Furthermore, following treatment with either 5FU or MTX, we observed that the number of cells in the G0/G1 phase decreased and those in the S phases increased. However, these cell cycle alterations were prevented by the addition of OG-VI. With the exception of 6MP-treated cells, we did not observe any effects on proliferation or cell cycle regulation that could be ascribed to the presence of Gln. Thus, we have demonstrated that OG-VI rescues cells from the cytotoxic effects of several chemotherapeutic agents. *J. Med. Invest.* 54 : 235-242, August, 2007

Keywords : exogenous nucleotides, 5-fluorouracil, glutamine, OG-VI, methotrexate, 6-mercaptopurine

INTRODUCTION

Nucleosides and nucleotides (NS/NT) are essential components of cells, involved in DNA and RNA synthesis, as well as protein metabolism (1). Mammalian cells can synthesize these components via *de novo* pathways and thus, deficiencies do not occur under normal conditions (1). In the clinical field,

nucleic acids are known to exert profound influence on nitrogen balance and the immune system (2-4). Exogenous applications of NS/NT are incorporated well into somatic cells such as brain tissue, and cells that undergo rapid-turnover e.g. those involved in repair or intestinal tissue (5, 6). The capacity of intestinal cells for synthesis of NT *de novo* is limited by high cell turnover rates (7), as this process requires significant amounts of energy. However, the salvage pathway requires less energy and thus, can supplement *de novo* generation in situations requiring increased synthesis of DNA and RNA (8).

OG-VI is a well-balanced NS/NT mixture comprising 30 mM inosine, cytidine and guanosine

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monophosphate, as well as 22.5 mM uridine and 7.4 mM of thymidine. In the clinical setting, OG-VI is delivered parenterally (9) and in animal models, it has been shown to increase protein synthesis and improve nitrogen balance following a partial hepatectomy (9, 10), as well as enhance hepatic DNA synthesis (11). OG-VI has been shown to support intestinal mucosal growth via parenteral nutrition (12, 13) following massive bowel resection (14) and during fetal small bowel transplantation (15).

In clinical practice, chemotherapy is a highly effective treatment for neoplasia. However, the chemotherapeutic agents can cause serious side effects in the gastrointestinal tract, since they are cytotoxic for normal, rapid-turnover tissues. The suffering caused by these side effects often limits the length of therapy (16, 17), and there has been no way to avoid this problem until now.

Glutamine (Gln) is the most abundant amino acid and it is conditionally essential during critical phases or following intestinal damage, since it is used by rapid-turnover cells as an important N-source for NT synthesis (18). Although several authors have demonstrated the advantages of Gln supplements in animals, showing that it suppresses the intestinal toxicity of treatment with chemotherapeutic agents, its therapeutic benefits remain unclear (19-23). In an *in vitro* study, Yamauchi, *et al.* reported Gln did not function as an energy source but as an N-donor for cellular NT synthesis, replacing exogenous NS/NT during proliferation or growth (24).

In this study, we used an *in vitro* model to estimate the benefits of exogenous OG-VI treatment on intestinal toxicity induced by three chemotherapeutic agents: 5-fluorouracil (5FU), a thymidine synthetase inhibitor that blocks *de novo* pyrimidine synthesis; methotrexate (MTX), a folate antagonist that blocks dihydrofolate reductase and thymidine synthetase; and 6-mercaptopurine (6MP), an inhibitor of *de novo* purine synthesis that depletes cellular levels of adenine, guanine and ribonucleotides. Furthermore, we evaluated whether or not the sensitivity of intestinal cells to chemotherapeutic agents varied under conditions of Gln deficiency and OG-VI supplementation.

MATERIALS AND METHODS

Reagents and Chemicals

OG-VI comprises 30 mM inosine, cytidine and guanosine monophosphate, 22.5 mM uridine and

7.4 mM thymidine. The total concentration of NS/NT in stock OG-VI was 120 mM and the final concentration of the OG-VI was 1.2 mM (1 : 100 dilution) in after the experiments. 5FU, MTX and 6MP were purchased from Wako Pure Chemical Industries (Osaka, Japan). Stock 5FU (100 μ M) was dissolved in PBS. Stock MTX (5 μ M) and 6MP (1 mM) were dissolved in 10 and 2 mM of NaOH, respectively.

Cell Culture

Caco-2 human intestinal epithelial cells were maintained in DMEM (Sigma), supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotic (50 μ g mL⁻¹ gentamycin). Cells were grown at 37°C in a humidified 5% CO₂/95% air atmosphere. For viability assays, cells (2×10⁴ cells/well) were seeded into 24-well plates in media with or without Gln, and containing 0.1% antibiotics, 0.1% MITO⁺ serum extender (Becton Dickinson) and 1% modified Eagle medium (MEM) non-essential amino acids solution (NEAA). Immediately after plating, OG-VI (1.2 mM) was added to the appropriate wells and cells were incubated at 37°C for 24 h, after which chemotherapeutic agents (1 μ M 5FU, 50 nM MTX or 10 μ M 6MP) or vehicle were added and the incubation continued for a further 7 days. Cell numbers were evaluated daily. Final OG-VI concentration (1.2 mM) was selected according to the optimal dose in *in vitro* study (1.2~0.006 mM) (24) and the blood concentration (5 mM) in *in vivo* study (9). Drug concentrations were selected according to recognized treatment levels. PBS and NaOH were used as vehicle controls.

Cell Viability Assay

Following treatment, Caco-2 cells were harvested by trypsinization and viability was measured using the trypan blue dye exclusion test, as described previously (24).

Cell Cycle Analysis

Caco-2 cells (1×10⁵ cells/well) were seeded into 6-well plates in the presence or absence of OG-VI (1.2 mM), then treated with 5FU (1 μ M), MTX (50 nM), 6MP (10 μ M) or the vehicle controls (PBS and NaOH). After 72 h treatment, cells were washed twice with PBS, harvested by trypsinization, fixed with ice-cold 70% ethanol, treated with RNase A (50 μ g mL⁻¹) and stained with propidium iodide (50 μ g mL⁻¹). Samples and data were analyzed further on a Beckman Coulter Epics XL-MCL. Calculations

lation of cell cycle distribution was performed using the Multi Cycle V. 2.53 software package (Phoenix Flow Systems, CA).

Statistical analyses

Data were expressed as the mean ± S.D. Statistical analyses were performed using the Student's t-test and *P*-values <0.05 were considered significant.

RESULTS

OG-VI promotes cell survival in 5FU-treated Caco-2 cells

In the presence and absence of Gln, OG-VI supplements recovered the inhibition of Caco-2 cell growth caused by 1 μM 5FU treatment (Fig. 1). Following 5FU treatment, the number of cells in the G0/G1 and S phases decreased and increased, respectively (Fig. 2). OG-VI supplements enabled re-

covery of the cell cycle, with progression of treated cells resembling that of the vehicle control. The effects of OG-VI were unaltered by Gln supplements.

OG-VI promotes cell survival in MTX-treated Caco-2 cells

In the presence and absence of Gln, OG-VI supplements recovered the suppression of Caco-2 cell growth caused by 5nM MTX treatment (Fig. 3). In the presence of Gln, some growth of MTX-treated cells was observed. Following MTX treatment, the number of cells in the G0/G1 and S phases decreased and increased, respectively. OG-VI supplements enabled recovery of the cell cycle, with progression of treated cells resembling the vehicle control (Fig. 4). The effects of OG-VI were unaltered by Gln supplements.

OG-VI promotes cell survival in 6MP-treated Caco-2 cells

Although we observed a small number of Caco-2

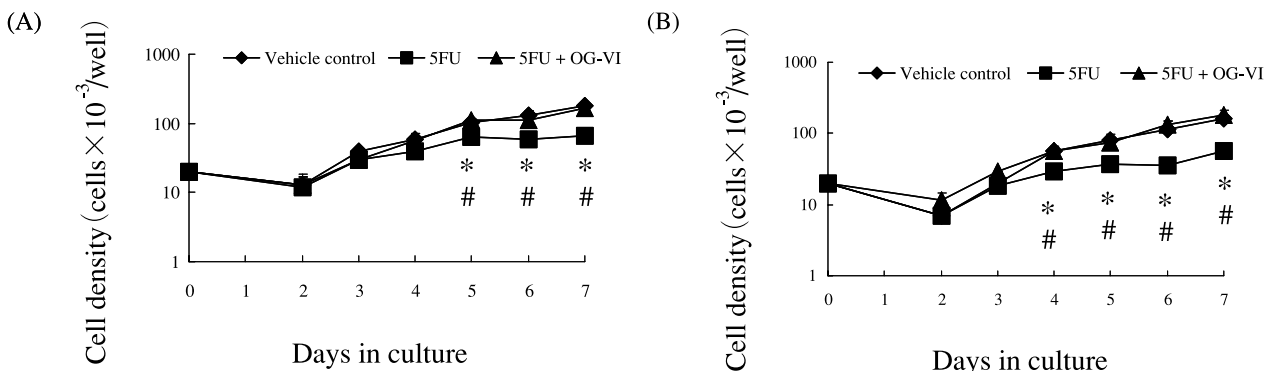


Fig. 1. Effects of OG-VI on the cell cytotoxicity of 5FU-treated Caco-2 cells. Cells were grown in culture media with or without OG-VI for 24 h, after which either 1 μM 5FU or vehicle control was added. (A) Media containing 4 mM Gln. (B) Gln-free media. All media contained 0.1% MITO⁺ serum extender and 1% NEAA solution. Values indicate means ± SD from three independent determinations. * Significant differences: *, compared to the vehicle control (*P* < 0.05); #, compared to 5FU + OG-VI (*P* < 0.05).

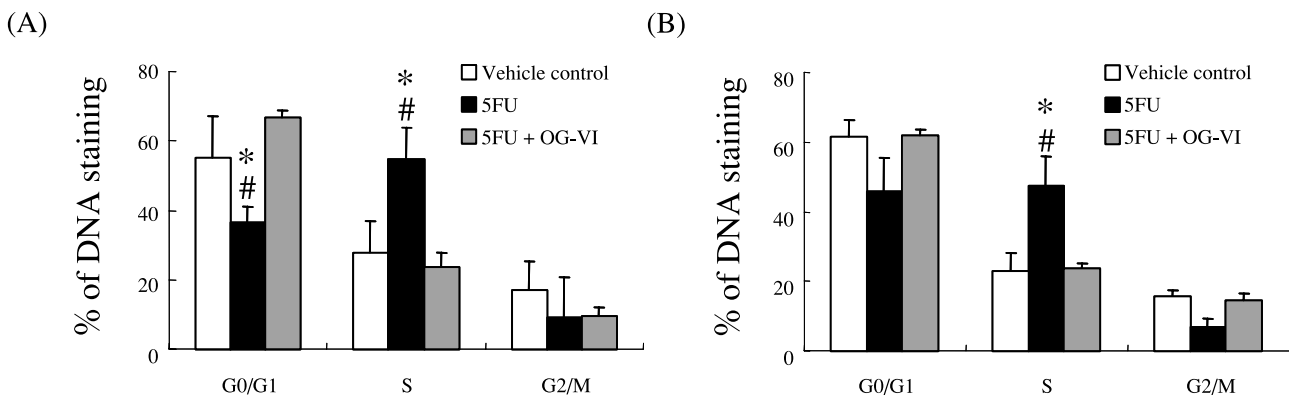


Fig. 2. Effects of OG-VI on the cell cycle distribution of 5FU-treated Caco-2 cells. Cells were grown in culture medium with or without OG-VI for 24 h after which either 1 μM 5FU or vehicle control was added. Cells were incubated for a further 3 days, prior to cell cycle analysis. (A) Media containing 4 mM Gln. (B) Gln-free media. All media contained 0.1% of MITO⁺ serum extender and 1% NEAA solution. Values indicate means ± SD from three independent determinations. Significant differences: *, compared to the vehicle control (*P* < 0.05); #, compared to 5FU + OG-VI (*P* < 0.05).

cell growth following treatment with 10 μ M 6MP, the addition of OG-VI returned growth to similar levels as observed for the vehicle control. In Gln-deficient conditions, the growth of OG-VI-supplemented cells was significantly greater than that of the vehicle

control ($P < 0.05$; Fig. 5). We observed no significant difference in cell progression following 6MP treatment, suggesting that this agent does not affect the regulation of cell cycling (Fig. 6).

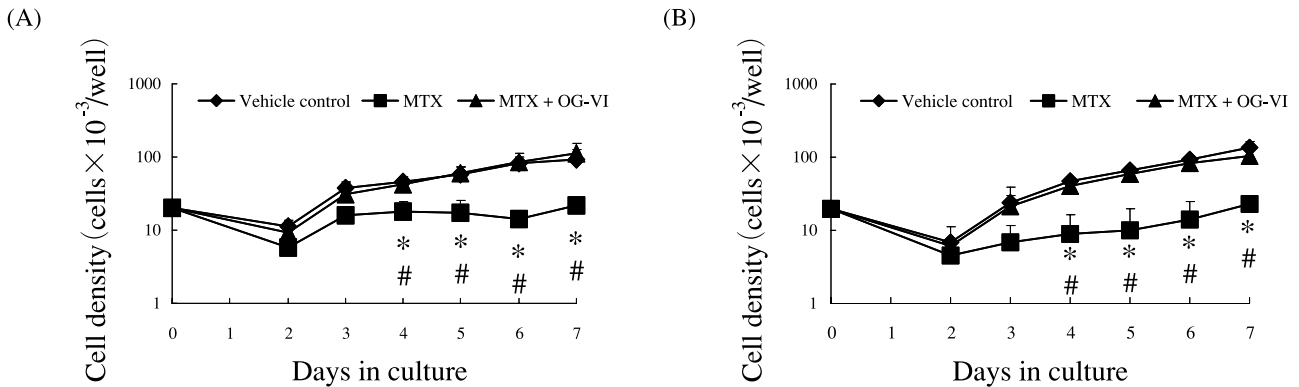


Fig. 3. Effects of OG-VI on the cell cytotoxicity of MTX-treated Caco-2 cells.

Cells were grown in culture medium with or without OG-VI for 24 h, after which either 5 nM MTX or vehicle control was added. (A) Media containing 4 mM Gln. (B) Gln-free media. All media contained 0.1% MITO⁺ serum extender and 1% NEAA solution. Values indicate means \pm SD from three independent determinations. Significant differences: *, compared to the vehicle control ($P < 0.05$); #, compared to MTX + OG-VI ($P < 0.05$).

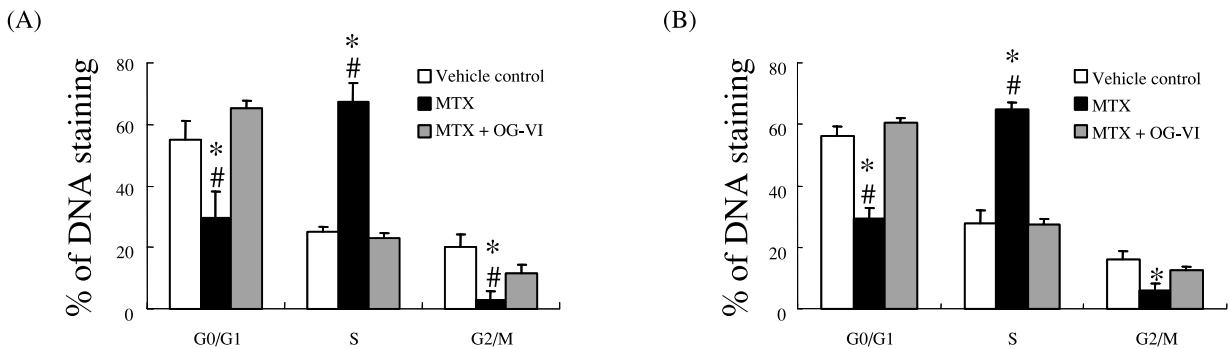


Fig. 4. Effects of OG-VI on the cell cycle distribution of MTX-treated Caco-2 cells.

Cells were grown in culture medium with or without OG-VI for 24 h, after which either 5 nM MTX or the vehicle control was added. Cells were incubated for a further 3 days, prior to cell cycle analysis. (A) Media containing 4 mM Gln. (B) Gln-free media. All media contained 0.1% of MITO⁺ serum extender and 1% NEAA solution. Values indicate means \pm SD from three independent determinations. Significant differences: *, compared to the vehicle control ($P < 0.05$); #, compared to MTX + OG-VI ($P < 0.05$).

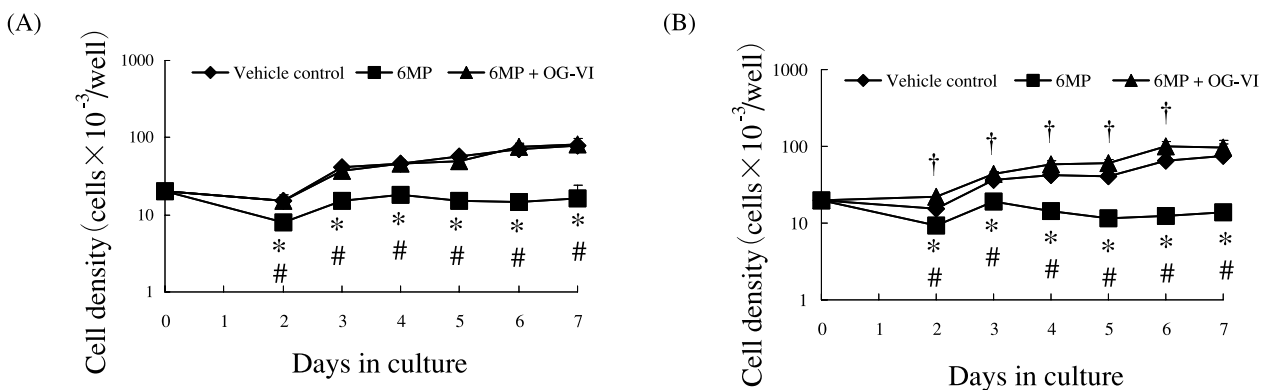


Fig. 5. Effects of OG-VI on the cell cytotoxicity of 6MP-treated Caco-2 cells.

Cells were grown in culture medium with or without OG-VI for 24 h, after which either 10 μ M 6MP or the vehicle control was added. (A) Media containing 4 mM Gln. (B) Gln-free media. All media contained 0.1% MITO⁺ serum extender and 1% NEAA solution. Values indicate means \pm SD from three independent determinations. Significant differences: *, \dagger , compared to the vehicle control ($P < 0.05$); #, compared to 6MP + OG-VI ($P < 0.05$).

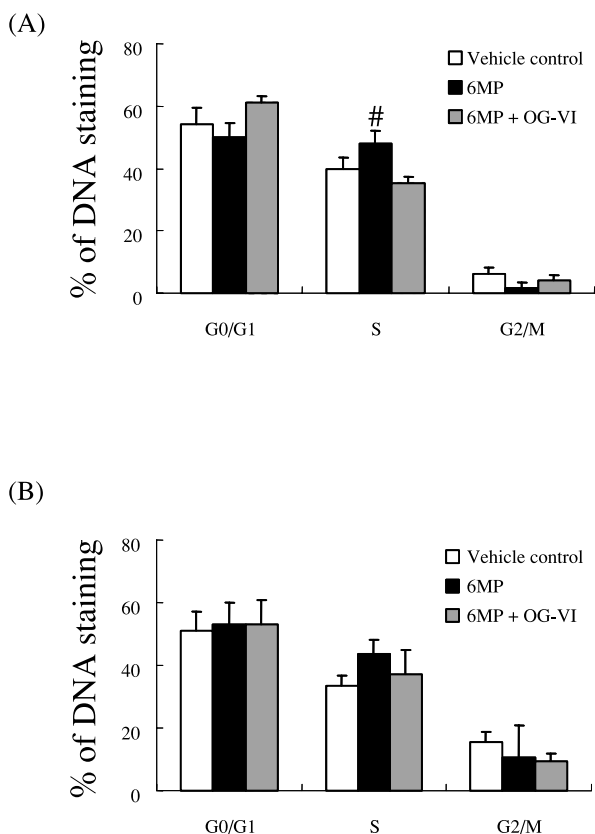


Fig. 6. Effects of OG-VI on the cell cycle distribution of 6MP-treated Caco-2 cells.

Cells were grown in culture medium with or without OG-VI for 24 h, after which either 10 μ M 6 MP or the vehicle control was added. Cells were incubated for a further 3 days, prior to cell cycle analysis. (A) Media containing 4 mM Gln. (B) Gln-free media. All media contained 0.1% MITO⁺ serum extender and 1% NEAA solution. Values indicate means \pm SD from three independent determinations. Significant differences #: compared to 6MP + OG-VI ($P < 0.05$).

DISCUSSION

In this study, we used the intestinal epithelial cell line Caco-2 to examine the effects of supplementation with an NS/NT mixture (OG-VI) on cytotoxicity caused by several anti-metabolites used in cancer chemotherapy. Since it is likely that *de novo* NS/NT synthesis is limited in cells treated with anti-metabolites, their exogenous addition might enhance Caco-2 cell proliferation. Previously, He, *et al.* reported that no enhancement of Caco-2 cell proliferation was observed in Gln-rich conditions (2 mM Gln) (25) although exogenous NTs enhanced proliferation in Gln-deficient conditions. Similarly, Sato *et al.* reported that addition of exogenous NTs did not enhance Caco-2 cell proliferation in the presence of 4 mM Gln (26). These authors concluded that in Gln-deficient conditions, reduced *de novo* synthesis of NTs caused decreased DNA synthe-

sis. Our results indicate that in both Gln-rich and -deficient conditions, OG-VI enhances the proliferation of Caco-2 cells treated with anti-metabolites.

Wang, *et al.* (27) described the effects of OG-VI addition on a gastric cancer cell line (KATO III) incubated with 5FU and OG-VI for 3 days. They observed that relative to the non-supplemented condition, viable cell numbers dropped significantly in the OG-VI-supplemented samples. Although this result appears to be contradictory to our findings, it may reflect differences in the NS/NT consumption patterns of each cell line. As such, KATO III cells were predicted to consume more purine than pyrimidine NTs (27). Furthermore, the uptake of guanosine, inosine and uridine is efficient in fetal rat hepatocytes, whereas the uptake of thymidine and cytidine is not (28). Modulation of proliferation by exogenous NTs (AMP, GMP, IMP and CMP, used individually or as a mixture) has been examined in two intestinal epithelial cell lines (IEC-6 and HT29); only mixed NTs effected IEC-6 proliferation, whereas either the NTs mixture or AMP effected HT29 proliferation (29). However, CMP is considered the most effective nucleotide for IEC-6 proliferation, presumably because exogenous pyrimidines are used prior to purines during proliferation of some intestinal cell lines (26). Although we did not compare the effect of individual OG-VI components, we did observe that the complete mixture enhanced proliferation of all the anti-metabolite treated Caco-2 cells in this study. This result indicates that the cells are able to use the exogenous supplements effectively.

With the exception of 6MP-treated cells, we did not observe significant effects of Gln on the cell growth. In Gln-deficient conditions, the proliferation of 6MP-treated cells was significantly greater in the presence of OG-VI than the vehicle control alone. This result suggests that exogenous OG-VI has a greater influence on cell proliferation than Gln deficiency, providing effective support for cell proliferation via NT salvage synthesis. Sukemori, *et al.* (30) reported that in cultured leukemia cells, a 60% reduction in Gln level enhanced the cytotoxicity of 5FU but not MTX. However, as 6MP interferes with purine synthesis and Gln is an N-source for purines base, it might be expected that Gln-deficient conditions will have a greater effect on 6MP cytotoxicity than either 5FU or MTX (7).

5FU is generally believed to induce the G1-S phase arrest due to inhibition of thymidine synthetase (31), and MTX is considered to kill cells in

the S-phase via irreversible binding to dihydrofolate reductase (32). Thus, both of these anti-metabolites block the cell cycle in the S phase. In contrast, 6MP induces the G2-M phase arrest, inhibiting purine synthesis via increased methyl-thio-IMP levels (33). We demonstrated that treatment with either 5FU or MTX resulted in an increase in the percentage of cells in the S-phase. Furthermore, we showed that regulation of the cell cycle returned to normal following addition of OG-VI. However, we did not observe cell cycle differences between the different 6MP treatment conditions, although vehicle control and OG-VI-supplemented cells proliferated, whereas those treated with 6MP alone did not grow. Thus, 5FU- and MTX- treated cells exhibit a different pattern of cell cycle arrest than 6MP-treated cells. The presence of exogenous NT or OG-VI leads to an increased RNA and DNA content of murine or rat intestines (14, 34-36), leading the speculation that although *de novo* NT synthesis has been blocked by each of anti-metabolite, cell cycle regulation returns to normal as a consequence of DNA and RNA synthesis. Different studies have shown conflicting results with respect to the effects of exogenous purine or pyrimidine NS/NT on proliferation and cell cycle regulation. However, these differences may relate to the presence or absence of NT receptors on the cells used. Extracellular nucleotides exert their effects via ionotropic P2X receptors or G protein-coupled P2Y receptors, in which the different receptors are activated by distinct NTs, purines or pyrimidines (37). Thus, the variations in proliferation and cell cycle regulation reported by each study suggest that there are different receptors involved in each processes and additional experimentation will be required to elucidate these mechanisms.

Since we have shown that OG-VI supports proliferative cell growth, it is of importance to determine whether or not supplementation will accelerate tumor growth. In an *in vivo* study by Usami, *et al.* (38), enhanced tumor growth was not observed when Yoshida sarcoma was implanted into rats and supplemented with OG-VI. In addition, the authors co-administrated 5FU with the individual NS/NT components and determined that thymidine, uridine and inosine suppressed tumor growth effectively, whereas 5'-GMP and cytidine did not. Thus, the utilization of exogenous nucleotides varies with cell type and NS/NT mixture component, and it is clear that the responses of different cell types and organs to exogenous NS/NT supplementation require further investigation.

Taken together, our results indicate that exogenous OG-VI supports the proliferation and cell cycle regulation in cells cultured with several anti-metabolites used in cancer treatment. Its usage could rescue rapid-turnover cells, such as those of the intestine, from the side effects of cancer treatment and might enable patients to complete chemotherapy. Further investigations will need to examine the effectiveness and consumption patterns of exogenous OG-VI on other types of rapid-turnover cells, as well as on cancer cells.

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