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GTP Induces S-phase Cell-cycle Arrest and Inhibits DNA Synthesis in K562 Cells But Not in Normal Human Peripheral Lymphocytes

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Since differentiation therapy is one of the promising strategies for treatment of leukemia, universal efforts have been focused on finding new differentiating agents. In that respect, we used guanosine 5'-triphosphate (GTP) to study its effects on K562 cell line. GTP, at concentrations between 25-200 μM, inhibited proliferation (3-90%) and induced 5-78% increase in benzidine-positive cells after 6-days of treatments of K562 cells. Flow cytometric analyses of glycophorine A (GPA) showed that GTP can induce expression of this marker in more mature erythroid cells in a time- and dose-dependent manner. These effects of GTP were also accompanied with inhibition of DNA synthesis (measured by [^{3}H]-thymidine incorporation) and early S-phase cell cycle arrest by 96 h of exposure. In contrast, no detectable effects were observed when GTP administered to unstimulated human peripheral blood lymphocytes (PBL). However, GTP induced an increase in proliferation, DNA synthesis and viability of mitogen-stimulated PBL cells. In addition, growth inhibition and differentiating effects of GTP were also induced by its corresponding nucleotides GDP, GMP and guanosine (Guo). In heat-inactivated medium, where rapid degradation of GTP via extracellular nucleotidases is slow, the anti-proliferative and differentiating effects of all type of guanine nucleotides (except Guo) were significantly decreased. Moreover, adenosine, as an inhibitor of Guo transporter system, markedly reduced the GTP effects in K562 cells, suggesting that the extracellulr degradation of GTP or its final conversion to Guo may account for the mechanism of GTP effects. This view is further supported by the fact that GTP and Guo are both capable of impeding the effects of mycophenolic acid. In conclusion, our data will hopefully have important impact on pharmaceutical evaluation of guanine nucleotides for leukemia treatments.

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Introduction

Chronic myelogenous leukemia (CML) arises from the reciprocal translocation between the abl gene on choromosome 9 and the bcr gene on choromosome 22 in pluripotent hematopoietic stem cells (Deininger *et al*., 2000; O'Dwyer, 2000). The Bcr-Abl oncogene generated from this translocation encodes a $p210^{Bcr\text{-}ABL}$ protein which is involved in growth factor-independent proliferation and survival of myeloid progenitor cells as well as resistance to apoptosis (Deininger *et al*., 2000; O'Dwyer, 2000). During chronic phase of the disease, leukemic myeloid progenitors undergo excess proliferation, however, these cells differentiate and function normally. Over 3-5 years, the disease is followed invariably with a progressive loss of terminal differentiation and the disease terminates in acute leukemia known as blast crisis (Pane *et al*., 2002; Wong *et al.,* 2003). At present, CML therapies mostly include chemotherapies, α-interferon treatments and bone marrow transplantation as well as combination therapies (Deininger *et al*., 2000; Wong *et al*., 2003). Recently, some focus has been devoted to STI571 for treatment of CML (Druker *et al*., 2001). However, clinical application of STI571 has been associated with some adverse effects (Thiesin *et al*., 2000; Kawano *et al.,* 2004). Indeed, in many cases, targeting of cancer through chemotherapeutic approaches would eliminate most but not completely all cancer cells within the patient, thereby leading to relapse of the disease after suspending the treatment (Reya *et al.,* 2001). To solve these problems, several other alternative therapeutic methods have been so far evaluated. The differentiation-inducing therapy seems to be a promising therapeutical approach, especially in elderly patients who can not tolerate intensive chemotherapy or bone marrow transplantation (Hait *et al.,* 1993; Gorin *et al.,* 2000;

Sell, 2004). In fact, with differentiation therapy, cancer stem cells can be also forced toward pathway of terminal differentiation and eventual senescence, and therefore the possibility of cancer relapse can be reduced (Clarkson *et al.*, 2003; Cao and Heng, 2005). In that respect, several clinical trials have been done using differentiating agents alone or in combination chemotherapy in myelodysplastic syndromes or CML. However, none of these agents have therapeutic potency comparable to all-*trans*-retinoic acid (ATRA) in acute promyelocytic leukemia (APL) (Gorin *et al.*, 2000; Clarkson et *al.*, 2003). This leaves the area open for discovery of new generation of differentiating agents.

The human CML cell line (K562) has been established from the pleural extravasation of a patient with CML in blast crisis (Lozzio and Lozzio, 1977). It behaves as a pluripotent hematopoietic stem cell (Lozzio and Lozzio, 1977). In addition to abnormal Bcr/Abl gene, K562 cells have also mutated p53 gene. These combined mutations make K562 cells a suitable and worldwide model for *in vitro* studies of CML blast phase treatment (Law *et al.*, 1993). On the other hand, K562 cell line has been extensively used as a suitable *in vitro* experimental system for the study of differentiation potential of many compounds (Lozzio and Lozzio, 1977; Law *et al.*, 1993; Clarkson *et al.*, 2003). Indeed, a number of drugs are known to be capable of inducing the differentiation of K562 cells into different lineages. For example, K562 cells have been differentiated into megakaryocytes by phorbol 12-myristate 13-acetate (PMA), while, hemin, anthracyclines, cytosine arabinoside, dimethyl sulfoxide (DMSO) and butyric acid have been shown to cause erythroid differentiation and/or growth inhibition of K562 cells in vitro (Koeffler and Golde, 1980; Villeval *et al.,* 1983; Tetteroo *et al.,* 1984; Kawasaki *et al.,* 1996). Regardless of these achievements, clinical evaluation of these compounds have not been promising. For instance, clinical use of PMA is not recommended due to its tumor promoting activity (Amuth and Berenblum, 1976). Similarly, DMSO, despite its powerful differentiating capacity, is unsuitable for internal human use (Danilenko *et al*., 2001). Thus, universal efforts have been devoted to find new and potent differentiation inducers devoid of general toxicities.

In that respect, it was recently shown that non-toxic concentrations of GTP and the related nucleotides induced the differentiation of K562 cells as efficiently as the inducers previously mentioned, suggesting its possible efficiency in treatment of CML (Osti *et al*., 1997; Morceau *et al*., 2000). Following GTP treatment of K562 cells, a sharp increase in cytoplasmic hemoglobin content (a differentiation marker) was accompanied by an increase in γ-globin expression (Morceau *et al*., 2000). The increase in the level of γ-globin expression points to an additional potential of GTP, as a therapeutic agent, in other hematological disorders such as β-tallassemia (Osti *et al*., 1997; Morceau *et al*., 2000). Attain to these evidences, further investigations seems to be required for a better understanding of anti-proliferative and differentiating effects of this naturally-occurring compound in order to facilitate its use as a therapeutic drug. In this investigation we evaluated the effects of GTP on growth, cell cycle, DNA synthesis and differentiation of K562 cells. Human peripheral lymphocytes (PBL) were also subjected to GTP effects. Our findings showed that GTP inhibited growth and DNA synthesis and induced S-phase cell-cycle arrest and erythroid differentiation in K562 cells. Whereas, PBL cells were not affected in those respects. Our results also showed that extracellular metabolism of GTP to guanosine (Guo) and its consequent uptake by the cells seemed to be the main route by which GTP exerts its effects.

Materials and Methods

Materials. The cell culture medium (RPMI 1640) and penicillin-streptomycin were purchased from Gibco BRL (life technolologies, Paisley, Scotland). Fetal bovine serum (FBS) was purchased from Jahad Daneshgahi of Tehran (Iran). The culture plates were obtained from Nunc (Denmark). Phytohemagglutinin (PHA), propidium iodide (PI), mycophenolic acid (MPA) and benzidine were purchased from Sigma. Chem. Co. (Germany). Dimethylsulfoxide (DMSO), H_2O_2, sodium hydroxide (NaOH) and methanol were obtained from Merck (Germany). [^{3}H]-thymidine was purchased from Amersham Pharmacia Biotech (UK). Guanosine (Guo), guanosine 5-monophosphate (GMP), guanosine 5-diphosphate (GDP), guanosine 5-triphosphate (GTP) and adenosine (Ado) were purchased from Aldrich Chemical Co. Ltd (England). GTP, GDP and GMP were dissolved in aqueous solution and were stored at concentrations of 20 mM at −20°C. A 10 mM Guanosine stock solution was prepared in NaOH (0.1 N).

Cells and culture conditions. The human K562 cell line, obtained from Pasteur Institute of Iran (Tehran), was cultured in RPMI-1640 medium supplemented with FBS (10%, v/v), streptomycin (100 μg/ml) and penicillin (100 U/ml) in CO_2 humidified atmosphere at 37°C. Normal human peripheral lymphocytes (PBL) from healthy volunteers were obtained following centrifugation on ficoll gradient. Cells at interface were removed, washed with phosphate buffer saline (PBS), and resuspended in RPMI-1640 medium containing 10% FBS, 100 U/ml penicillin and 100 μg/ml of streptomycin. Phytohemagglutinin (PHA, 5 μg/ml) was added to PBL cells to induce their proliferation. Cell numbers and viability were assessed using a hemocytometer and the ability of the cells to exclude trypan blue. All the experiments (if not otherwise indicated) were performed in RPMI medium supplemented with 10% of native (not heat-inactivated) FBS (referred to as native medium) to allow extracellular nucleotide metabolism as in vivo (Weisman *et al.,* 1988). For cell culture incubated more than 3 days, the medium and GTP were renewed at the third day of cell culture. To deactivate the serum-derived enzymes, RPMI-1640 containing 10% FBS was heated for 1 h at 59°C (Schneider *et al.,* 2001) right before use followed by cooling to 37°C for cell culture application. All experiments have been done in triplicate.

Microscopic evaluation of hemoglobin-containing differentiated K562 cells. The rate of erythroid differentiation of K562 cells was

estimated by benzidine staining of hemoglobin producing cells. K562 cells were seeded at a density of 4×10^4 cells/ml in a 24-well plate and incubated in RPMI-1640 with 10% FBS and treated with different concentration of GTP or the related compounds for 1-6 days. Medium and GTP were renewed on the third day of treatment. At every 24 h, the cells were collected and stained with a solution containing 8.5 ml benzidine solution (1.5% in methanol), 15 ml ethanol and 6.5 ml H_2O_2 (30%) for 5 min. A minimum of 1000 cells were counted under a light microscope to determine the percent of benzidine positive (brown to orange) cells (Morceau *et al.,* 2000).

Flow cytometric assessment of GPA on erythroid cell surfaces. Erythroid differentiation was also studied by evaluating the expression of glycophorine A (GPA), a major transmembrane sialoglycoprotein on red blood cells and the erythroid precursors. GTP-treated K562 cells were washed twice with PBS and resuspended in PBS containing 2% FBS. Ten µl of the mouse anti-human FITC-conjugated GPA monoclonal antibody (IQ product) was added to 100 µl of the cell suspension. As a negative control for background staining, an isotype matching mouse IgG_1 was used for staining untreated and GTP-treated cells. After incubation on ice for 30 min, the cells were washed and resuspended in PBS. The fluorescence of the cells was then measured by flow cytometry (Partec Pas).

Cell cycle analyses of GTP-treated cells by flow cytometry. DNA content was analyzed by a flow cytometer (Partec PAS). The K562 and PBL cells (4×10^4 cells/well) were seeded into 24-well plates, 24 h prior to treatments. After treatment with different doses of GTP for various time intervals, the cells were harvested, washed twice with PBS, fixed in 70% ethanol, and kept at –20°C till analyses. The cells were then stained with 20 µg/ml propidium iodide containing 20 µg/ml RNase (DNase free) for 2 h. The stained cells were then analyzed by flow cytometry (Moosavi *et al.,* 2005). The population of G_0/G_1, S, and G_2/M were determined using Multicycle Cell Cycle Software. The results are expressed as percentage of the cells in each phase.

DNA synthesis. DNA synthesis was estimated by measuring [^{3}H]-thymidine incorporation as previously described (Voigt *et al.,* 2000). Briefly, K562 and PBL cells (4×10^4 cells/ml) were seeded per well and different concentrations of GTP were added to each well and incubated for various time intervals. [^{3}H]-thymidine (1 µCi/well) was included during the last 6 h. Cells were then precipitated twice with 1 ml of tricholoric acid (10%). Cells were pelleted, the precipitate was then dissolved in 0.4 M NaOH, and the radioactivity was determined in a liquid scintillation counter (Rackbeta, LKB) as a measure of thymidine incorporation into DNA.

Results

GTP inhibits growth and induces differentiation of K562 cells but not normal PBL cells. The exponentially growing K562 cells (4×10^4 cells/ml) were exposed to different concentrations of GTP for various time intervals. The effects of a single dose treatment of GTP on growth and viability of K562 cells were studied by trypan blue exclusion assay. At concentration of 25 µM, GTP had no inhibitory effects (3% ± 1.2) on proliferation of K562 cells whereas, at 50 µM, GTP inhibited K562 proliferation by 30% after 6 days. The cell growth inhibition increased by 70, 82 and 90% at GTP concentrations of 100, 150 and 200 µM, respectively (Fig. 1A). These effects were also time-dependent, so that 100 µM GTP induced 4-70% growth inhibition of K562 cells between 1-6 days (Fig. 1B). The viability of K562 cells remained high (96%) after 6 days of GTP treatment at concentrations of up to 200 µM, indicating that GTP shows a cytostatic rather than cytotoxic activity in K562 cells (Fig. 1A).

The GTP-induced inhibition of K562 cell growth was accompanied by the induction of differentiation. Benzidine staining was used as an easy and rapid assay for distinction between differentiated hemoglobin-containing (benzidine-positve) and undifferentiated (benzidine-negative) K562 cells. After 6-days of treatment with increasing concentration of GTP, the percentage of the differentiated cells increased to a plateau of 66-76% at GTP concentrations of equal or more than 150 µM (Fig. 1A). The differentiation effects of GTP were also time-dependent, so that 100 µM GTP induced 2-66% differentiation of K562 cells between 1-6 days (Fig. 1B). These results consisted with the time-dependent growth inhibitory effects of GTP and are also in accordance with Osti *et al.* observations (Osti *et al*., 1997; Morceau *et al*., 2000). The extent of differentiation in untreated K562 cells, during the same time period, was approximately 1-3%.

To further evaluate the effects of GTP on K562 cell differentiation, the expression of glycophorine A (GPA), as an erythroid-specific marker, was evaluated by flow cytometry. In untreated K562 cells, stained with FITC-conjugated GPA mAb, as many as 73% of the cells fell within the range of background fluorescence. This is in contrast to 36 and 24% of K562 cells which have been treated with 100 and 200 µM GTP for 6 days, respectively (Fig. 1C). The expression of GPA, as demonstrated by a shift in flow cytometric profile, was also increased by time, where about 31, 52 and 64% of the cells were GPA-positive after treatments with 100 µM GTP for 2, 4 and 6 days, respectively (Fig. 1D). Thus, our results indicate that the proportion of GPA-expressing cells increased in a time- and dose-dependent manner in the presence of GTP.

To determine whether growth inhibitory effects of GTP occur in normal cells, we investigated the effects of this nucleotide on human normal peripheral lymphocytes. Both unstimulated- and stimulated-PBL cells were treated with 100 and 200 µM GTP for various time intervals (Fig 2A). Interestingly, no detectable changes in cell numbers and the viability of unstimulated-PBL cells were observed. This is in contrast to significant increase in both the growth and the viability of the stimulated-PBL cells after GTP treatments (Fig. 2B).

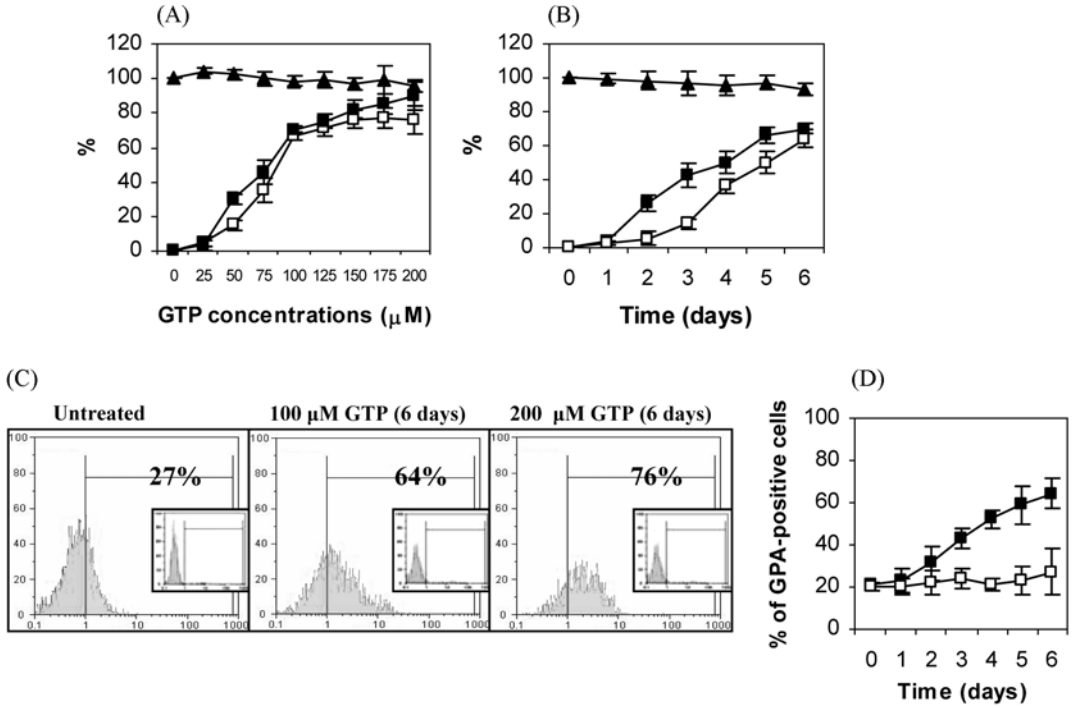

Fig 1. Time- and dose-dependent effects of GTP on proliferation, viability and differentiation of K562 cells. The cells were seeded in 24-well plate and treated with different concentrations of GTP for various time intervals. Medium and GTP were renewed on the third day of treatment. A: Effects of 25-200 μM GTP on growth, viability and differentiation of K562 cells after 6 day. B: Effects of 100 μM GTP on growth and differentiation of K562 cells at different time intervals. In A and B, (■) represents growth inhibition (% of control), (□) indicates % of benzidine-positive cells and (▲) represents viability (% of control). C: Effect of GTP on glycophorine A expression in K562 cells after 6 days of treatment with 0, 100 and 200 μM GTP. Control and treated K562 cells were stained with FITC-conjugated mouse anti-human GPA mAb. A FITC-conjugated irrelevant antibody was used as negative control for background fluorescence of untreated and GTP-treated K562 cells (insets). The green fluorescence of stained cells was measured by flow cytometry. D: Time-dependent expression of GPA in GTP (100 μM) treated K562 cells (■) compared to control untreated cells (□). The cells were harvested each day and the fluorescence of the cells was measured by flow cytometry as explained above. The results (A, B and D) are the average of triplicate countings ± SD ($p < 0.05$). The results shown in C are from a single typical experiment.

GTP induces S-phase arrest and inhibition of DNA synthesis in K562 cells. Our results clearly demonstrated that GTP had profound effects on K562 cell growth (Fig. 1A). In order to examine the effect(s) of GTP on cell cycle progression, we subjected the untreated and GTP-treated cells to flow cytometric analyses. Flow cytometry analyses of untreated cells, indicated that almost 58, 23 and 19% of the cells were distributed among G_0/G_{1-}, S-, and G_2/M-phases, respectively. After treatment of K562 cells with 100 μM GTP for 24 h, the cell cycle distribution remained comparable with that of control cells (Fig. 3A). However, longer exposures to GTP increased the percentage of K562 cells in S-phase (48 h, 34%; 72 h, 42% and 96 h, 49% compared to about 2-5% variation in control cells), meaning that after 24 h, the S-phase arrested K562 cells were unable to proceed into the G_2/M phase (Fig. 3A). A time-dependent increase in early S-phase cell population along with a compensated decrease of cells in G_0/G_1 and G_2/M phases were observed by 96 h of treatment. However, after 96 h, a plateau in percentage of S-phase cells was obtained. In addition, accumulation of cells in early S-phase could be seen after application of 50, 150 and 200 μM of GTP (Fig. 3B). As depicted in Fig. 3B, at 200 μM GTP, the accumulated cells in early S-phase are very close to G_1 peak and the populations of cells in G_0/G_1 and in G_2/M phases have significantly decreased. Treatment of K562 cells with 100 μM guanosine (Guo) resulted in a similar increase in S-phase cells (Fig. 3C). Despite these observations in K562 cells, the cell population pattern of unstimulated-PBL cells did not change during 4-6 days of GTP treatment and the cells remained in G_0/G_1 phase of their cell cycle (Fig. 3D), while an increase in S-phase population of GTP-treated and stimulated-PBL cells (to 39% compared to 24% of the corresponding control sample) was observed (Fig. 3D). However, this 15% increase in S-phase cells can be related to the effects of GTP on enhanced cell proliferation (Fig. 2) and DNA synthesis (Fig. 4B) in stimulated-PBL cells rather than to the S-phase arrest phenomena.

To clarify this matter, DNA synthesis in the GTP-treated cells was evaluated through [H^3]-thymidine incorporation into DNA. Figure 4A indicates that a time- and dose-dependent

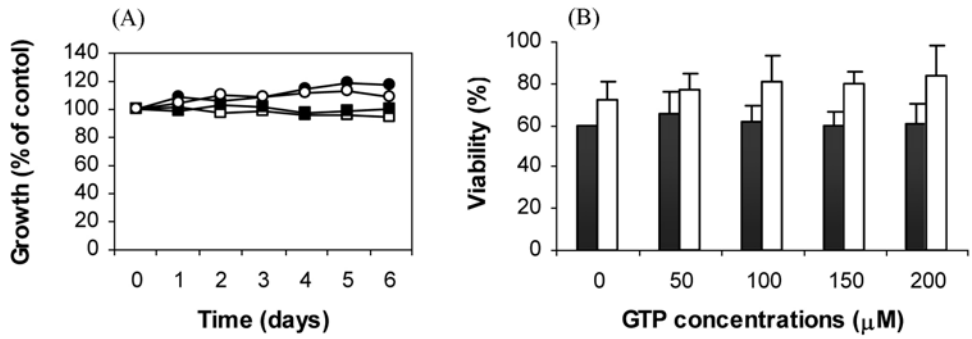

Fig. 2. The effects of GTP on proliferation and viability of unstimulated- and stimulated-PBL cells. The cells (4×10^4 cells/ml) were seeded in a 24-well plate with and without 5 μg/ml PHA for 24 h and then were treated with different concentrations of GTP for various time intervals. Medium GTP and PHA were renewed on the third day of treatment. A: Effects of 100 (○, □) and 200 (●, ■) μM GTP on cell number of unstimulated- (squares) and stimulated- (circles) PBL cells. The results are the means of four independent experiments and present as percent of respective untreated control cells. B: The effects of different concentrations of GTP on viability of unstimulated- (■) and stimulated- (□) PBL cells after 6 days. Viability was determined by trypan blue exclusion method and the results represent the average of three independent experiments ± SD ($p < 0.05$).

decrease in DNA synthesis was obtained when the K562 cells were treated with 100 and 200 μM GTP. However, consistent with results from the effects of GTP on PBL cell growth (Fig 2A and 3D, the extent of DNA synthesis did not varied in unstimulated-PBL cells up to 6 days of exposure to 100 μM GTP while the stimulated-PBL cells showed an increase in the extent of DNA synthesis (Fig. 4B). Indeed, careful examination of data clearly indicate that PHA was capable of inducing DNA synthesis through enhancing the extent of proliferation in stimulated-PBL cells (Fig. 2A) while, such an event was not recorded in unstimulated-PBL cells (data not shown).

GTP acts through guanosine in inducing growth inhibition and cell differentiation. Recent studies have shown that exogenous purine nucleotides added to cell culture media are mainly degraded by the serum-derived enzymes and to lesser extent by membrane-bound ectoenzymes of the cells (Schneider *et al.,* 2001). To clarify how GTP exerts its effects (in intact or degraded form(s)) we used either native (heat-untreated medium) or heat-treated medium to culture the cells (Weisman *et al.,* 1988). According to results presented in Table 1, guanine nucleotides as well as Guo are capable of inhibiting the cell growth. Inhibition of serum-derived enzymes by heat treatment (59°C for 60 min) led to significant reduction in the growth inhibitory and differentiating effects of GTP, GDP and GMP, whereas Guo effects were slightly increased. Cell growth and viability of the control samples were not significantly altered using heat-inactivated media compared to native media (data not shown). Based on these observations, we can conclude that the effects of GTP on growth and differentiation of K562 cells is carried out through its conversion to Guo. In that case, inhibition of Guo uptake process would block all the biological functions reported for GTP. To evaluate this hypothesis, we studied the effects of adenosine, as a competitive nucleoside transport inhibitor (Cass *et al.,* 1999), on the growth inhibitory and differentiating activities of GTP in K562 cells. The results revealed that inhibition of Guo uptake was accompanied with a time-dependent decrease in growth inhibitory and differentiating effects of GTP (Fig. 5). Indeed, co-treatment of K562 cells with GTP (100 μM) and adenosine (100 μM) reduced growth inhibition (Fig. 5A) and differentiation (Fig. 5B) effects of GTP by 42 and 43% after 6-days, respectively. Adenosine itself caused an 18 ± 2.2% (n = 3, $p < 0.05$) decrease in cell number and a 12 ± 1.6% (n = 4, $p < 0.05$) increase in number of benzidine-positive cells after 6-days of treatment of K562 cells (data not shown). An additional experiment with mycophenolic acid (MPA), a potent inhibitor of inosine monophosphate dehydrogenase (IMPDH), supported that the uptake of Guo has been inhibited by Ado. We designed this experiment based on the well-known role of extracellular Guo in impeding the anti-proliferative and differentiating effects of IMPDH inhibition (Batiuk *et al.,* 2001). Indeed, IMPDH is the rate limiting enzyme in biosynthesis of GTP and its inhibition results in GTP depletion and consequently, profound effects on different cell functions such as differentiation and apoptosis (Jayaram *et al.,* 1999). However, Guo, in concentrations to replete the GTP pool size through salvage pathway, can inhibit the anti-proliferative and differentiating effects of IMPDH inhibition (Jayaram *et al.*, 1999). The results in Table 2 clearly indicate that both Guo and GTP impeded the anti-proliferative and differentiating effects of IMPDH inhibitor (MPA). As expected, the protective effects of Guo and GTP were impaired by Ado, suggesting the requirement of Guo uptake for both processes. Taken together, the results indicated that the growth inhibitory and differentiating activities of GTP were mediated through generation and uptake of Guo.

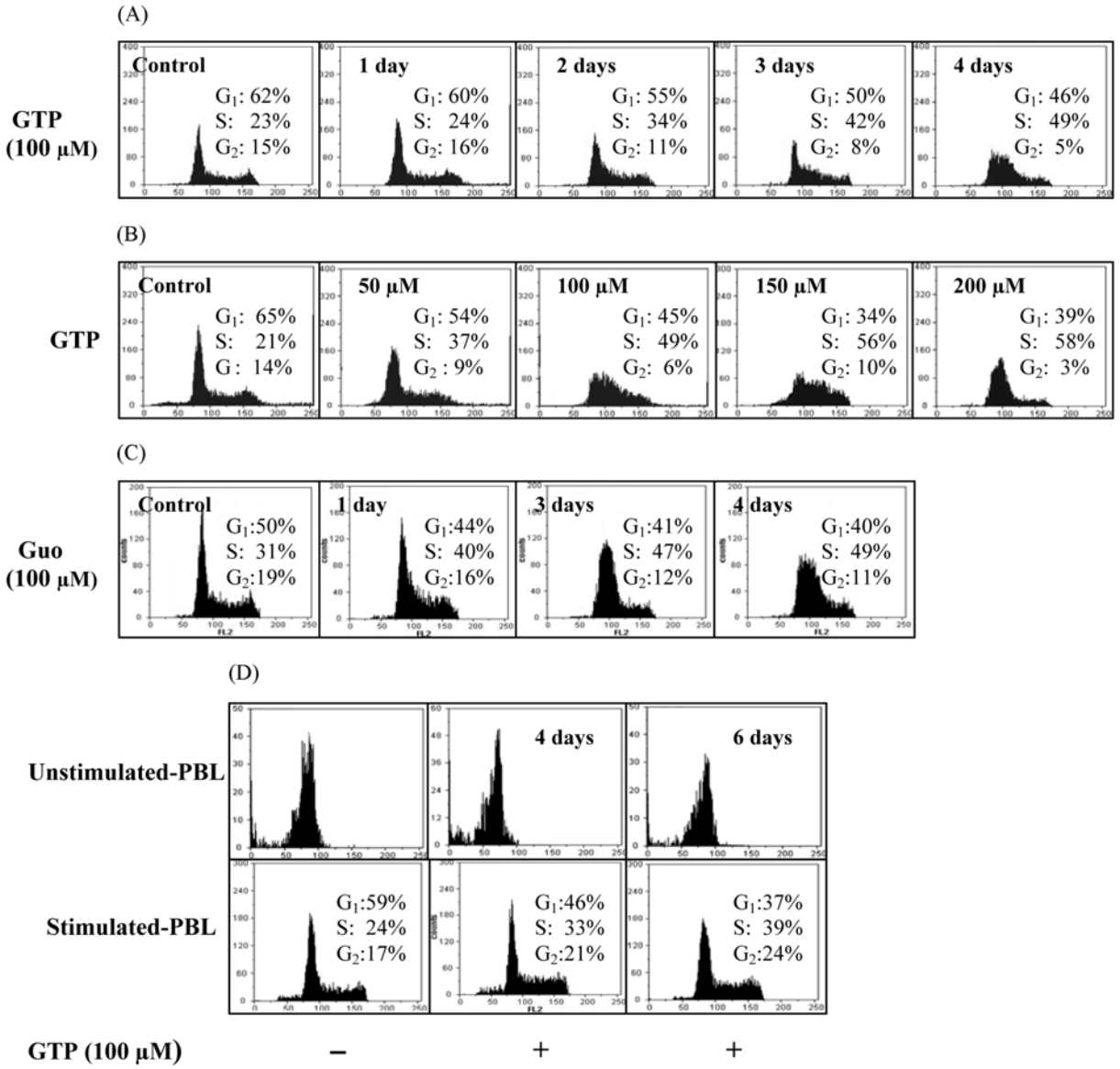

Fig. 3. The effects of GTP and Guo on cell cycle progression of K562 (A, B and (C) and normal PBL (D) cells. A: Time-dependent effects of GTP (100 μM) on K562 cells. B: Dose-dependent effects of GTP on K562 cells. The cells were treated with 50, 100, 150 and 200 μM GTP for 4 days. C: Time-dependent effects of Guo (100 μM) on K562 cells. D: Cell cycle effects of GTP in unstimulated- and PHA-stimulated-PBL cells. Unstimulated- and stimulated-PBL cells were treated with 100 μM GTP for 4 to 6 days and then analyzed by flow cytometry. The percentages of the cells in each phases of the cell cycle were determined by flow cytometry analyses as described in materials and methods. The results are those of a typical experiment.

Discussion

Among possible biological response modifiers, extracellular purine nucleotides and nucleosides have been the center of focus due to their established role in many physiological events such as inhibition of platelet aggregation, immunomodulation, cardiovascular effects and neurotransmission (Schneider *et al.,* 2001; Moosavi *et al.,* 2005). In the past few years, high interest has been devoted to their cytotoxic and differentiation capabilities in many transformed and non-transformed cell lines (Rapaport, 1983; Rapaport, 1994; Chow *et al.,* 1997; Rathbone *et al.,* 1999; Schneider *et al.,* 2001; Lemoli *et al.,* 2004). Among purines, it has been reported that extracellular guanine nucleotides could inhibit growth and induced differentiation of a variety of cells, such as human PC12 neuronal (Rathbone *et al.,* 1999), B16 melanoma (Giotta *et al.,* 1978) and K562 leukemia (Osti *et al.,* 1997) cell lines. To get a better understanding of the subject, we investigated, in a more detailed approach, the differentiation and anti-proliferative effects of GTP and the other related derivatives.

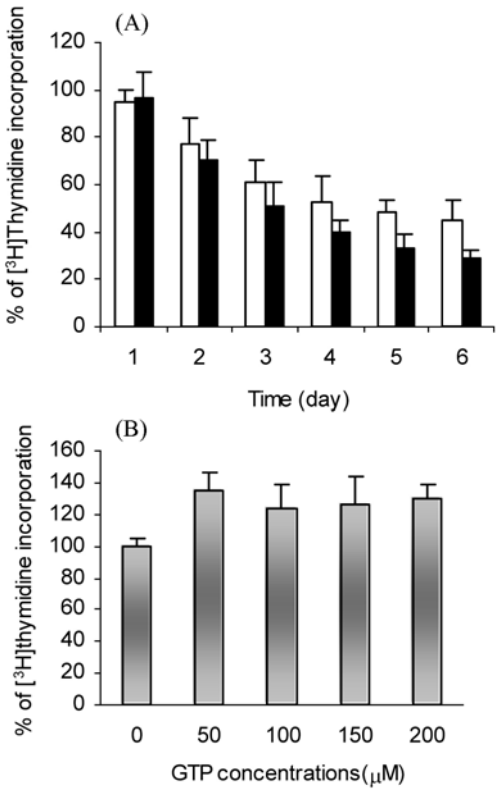

Fig 4. The effects of GTP on [^{3}H]-thymidine incorporation in K562 (A) and normal PBL (B) cells. A: The K562 cells (4×10^4 cells/ml) treated with 100 (□) or 200 (■) μM GTP for various time intervals. [^{3}H]-thymidine (1 μCi/well) was added to each sample six hours before the cell harvestings. The cells were then counted and equal number of cells was used for DNA analyses. B: The PBL cells (4×10^4 cells/ml) were seeded in 24-well plate and were stimulated with 5 μg/ml PHA for 24 h, then they were treated with different concentrations of GTP for 6 days. DNA synthesis was measured in terms of [^{3}H]-thymidine incorporation with respect to GTP-untreated control cells. The results are from three independent experiment ± SD ($p < 0.05$). The extent of inhibition of DNA synthesis was determined using the following equation: % inhibition = 1 – [test (cpm)]/[control (cpm)] × 100 and was represented as percent of respective untreated control cells.

According to our results, GTP showed time- and dose-dependent anti-proliferative and differentiation effects. The expression of GPA, the marker of more matured erythroid cells, was also increased in a time- and dose-dependent manner, suggesting terminal erythroid differentiation. It is believed that the expression of GPA first appears at the basophilic normoblast stage of erythroid development followed by higher expression in the more matured erythroid cells (Moore *et al.,* 1991). This is in contrast to most of the other differentiation-inducers of K562 cells which are only involved in early stages of erythroid differentiation (Kawasaki *et al.,* 1996).

The effects of GTP on normal and stimulated-PBL cells were also evaluated. According to our results, the healthy-unstimulated PBL cells were totally unaffected by GTP whereas the stimulated healthy PBL cells showed an increase in proliferation with no evidence for cell death. Although our data with PBL cells have tight correlation with the trophic effects of GTP in several normal neuronal cells, different observations have been obtained using neoplastic cells, such as human melanoma B16, erythroid leukemia K562 and T-cell leukemia Jurkat cells (Batiuk *et al.,* 2001). Whether these differences simply reflect the quantitatively smaller effect of GTP in PBL cells compared to K562 cells or a true phenotypic difference between normal and malignant cells are unknown. Nevertheless, significant selective effects in neoplastic cells compared to normal ones open up the chance to candidate this naturally-occurring compound as a therapeutic agent in cancer treatments.

According to literature, cell cycle arrest may be a prerequisite step for initiating terminal differentiation (Bernhard *et al.,* 2000). Although, G_1 arrest has been the center of attention in differentiation, some reports are concerned with the involvement of G_2 and S-phase arrest in this event (Rapaport, 1983; Gorin *et al.,* 2000). In our investigation, an early S-phase arrest was documented under GTP and Guo treatments in K562 cells followed by their differentiation. These observations are in full agreement with recent reports concerning cell cycle effects caused by purine nucleotides and nucleosides in CHO and BHK mammalian cells (Carlile *et al.,* 2004). Interestingly, DNA synthesis, a hallmark of S-phase progression was also negatively regulated in GTP-treated K562 cells. Inhibition of DNA synthesis was accompanied by the cell differentiation suggesting that duplication of the cellular genome during the S-phase of cell cycle is a critical event during which the cells are highly susceptible to the induction of differentiation (Plagemenn *et al.,* 1975; Rapaport, 1983; Bernhard *et al.,* 2000; Huang *et al.,* 2002). Consisted with this concept, differentiation can be induced in K562 cells by purines (Plagemenn et al., 1975; Rapaport, 1983; Huang et al., 2002) or other compounds (Hatse *et al.,* 1999; Bernhard *et al.,* 2000) that affect nucleotide metabolism and consequently the DNA synthesis, but not probably by agents affecting mitosis (e.g. vinblastine) or interfering with RNA or protein syntheses (Bernhard *et al.,* 2000). Exposure of K562 cells to phosphonate 9-(2-phosphonyl-methoxyethyl) adenine, an inhibitor of DNA synthesis, resulted in a similar S-phase arrest and erythroid differentiation (Hatse *et al.,* 1999). We therefore proposed that the inhibition of a key enzyme in biosynthesis of purines may be involved in inhibition of DNA synthesis, S-phase arrest and consequently differentiation of K562 cells.

Based on results presented in Table 1, GTP may exerts its effects after hydrolysis to guanosine since GDP and GMP were also very effective in inhibiting the proliferation, inducing of S-phase cell cycle arrest, and enhancing the differentiation of K562 cells. In addition, we showed that the anti-proliferative and differentiating effects of GTP, GDP and

Table 1. Cytostatic and differentiating effects of guanine nucleotides and guanosine on K562 cells in native medium (RPMI-1640 supplemented with 10% native FBS) or inactivated medium (heat inactivation) after 6 days. All guanine nucleotides as well as Guo have been renewed at the third day of treatments. Data are from three independent experiments ± SD

Medium	Treatment	Growth inhibition (% of control)	% of benzidine-positive cells
Untreated medium (RPMI 10% FBS)	Control	0	2 ± 1
	GTP (100 μM)	70 ± 3	66 ± 2
	GDP (100 μM)	65 ± 2	50 ± 3
	GMP (100 μM)	74 ± 5	63 ± 5
	Guo (100 μM)	60 ± 3	55 ± 4
Heat-inactivated medium (60 min, 59°C)	Control	0	3 ± 1
	GTP (100 μM)	11 ± 2	16 ± 3
	GDP (100 μM)	14 ± 3	9 ± 6
	GMP (100 μM)	19 ± 2	13 ± 5
	Guo (100 μM)	75 ± 5	73 ± 4

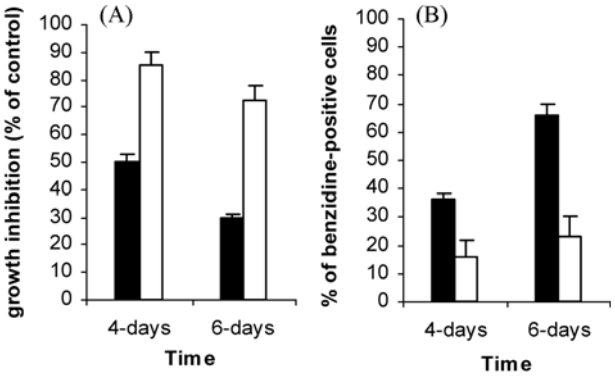

Fig 5. Effects of adenosine (as guanosine uptake inhibitor) on growth (A) and differentiation (B) of K562 cells. The cells were treated either with 100 μM GTP alone (■) or with a combination of 100 μM Ado and 100 μM GTP (□). The state of cell growth (A) and the extent of cell differentiation (B) were evaluated after 4 and 6 days of treatments. Medium, GTP and Ado have been renewed at the third day of the experiment. The results are from three independent experiment ± SD ($p < 0.05$).

Table 2. Effects of GTP, Guo and Ado on anti-proliferative and differentiating effects of an IMPDH inhibitor, MPA. K562 cells were treated with MPA (3 μM) for 3-days. The cell numbers and the extent of differentiation were established by trypan blue exclusion test and the benzidine assay, respectively. Data are from three independent experiments ± SD ($p < 0.05$)

Compound	Growth inhibition (% of control)	% of benzidine-positive cells
MPA	79 ± 3	65 ± 4
MPA + Ado	82 ± 5	73 ± 2
MPA + Guo	15 ± 6	9 ± 4
MPA + GTP	19 ± 5	17 ± 3
MPA + Guo + Ado	67 ± 2	55 ± 4
MPA + GTP + Ado	60 ± 7	51 ± 6

GMP are mainly mediated by their common metabolite, Guo. In the heat-inactivated medium, where rapid degradations of GTP via extracellular nucleotidases are quenched or slowed down, the anti-proliferative and differentiating effects of all nucleotides (but not Guo) were significantly decreased. These observations are in full contrast to results reported by Osti *et al.* (Osti *et al*., 1997) and Moreason *et al.* (Morceau *et al*., 2000) who have categorize anti-proliferative and differentiating properties for GTP in the heat-inactivated media. These results may arise due to incomplete heat-inactivation of serum derived enzymes. Indeed, GTP is also converted to guanosine extracellulary by a series of ectoenzymes which include a nucleoside triphosphatase, nucleoside diphosphate phosphohydrolase and 5'-nucleotidase. As these enzymes have variable kinetics (Gysbers and Rathbone, 1996; Rathbone *et al.,* 1999), it would be unlikely that GTP has been converted stochiometrically to Guo. On the other hand, it is believed that the free Guo added to the system is more prone to metabolism to guanine and/or deaminated product(s) than Guo derived from GTP, GDP and GMP. These events will certainly lead to lesser biological effects of Guo compared to Guo derived from GTP at equimolar concentrations (Gysbers and Rathbone, 1996; Osti *et al.,* 1997; Rathbone *et al.,* 1999). A similar situation has been reported on cytotoxicity of exogenous adenosine in human leukemia cells compared to adenosine derived from ATP or cAMP (Schneider *et al.,* 2001). Adenosine produced from degradation of ATP or cAMP is less prone to deamination by adenosine deaminase than free adenosine added directly to the medium. Based on these documents, it is expected that GTP will show stronger and longer lasting anti-proliferative and differentiating effects relative to Guo at a given dose.

In conclusion, our results showed that GTP administration to K562 cells inhibited their growth and DNA syntheses followed by induction of S-phase arrest and differentiation. In search of the mechanism of action, our data support the view that GTP exerts its effects through continuous degradation to

Guo by the assisting elements of the growth medium and probably the cell surface enzymes.

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