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INDUCTION OF ERYTHROID DIFFERENTIATION OF K562 CELLS BY 4-CARBAMOYLIMIDAZOLIUM 5-OLATE (SM-108)

YUKI HATA, YASUFUMU YAMAJI, TAIICHI SHIOTANI, JIRO FUJITA, HIROSHI KAMANO, KAZUMA IKEDA, JIRO TAKAHARA and SHOZO IRINO*

First Department of Internal Medicine, Kagawa Medical School, Kagawa 761-07, Japan

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Abstract—The effects of 4-carbamoylimidazolium 5-olate (SM-108), an antipurine compound, on a human leukemia cell line, K562, were studied. Treatment with SM-108 induced erythroid differentiation of K562 cells. During a 6-day culture with 100 μM SM-108, the cell number decreased to 37% of the control number, 77% of the cells became benzidine-positive, and the hemoglobin content increased from 2.1 ± 0.2 to 10.6 ± 1.3 pg/cell. Cell differentiation was associated with reduction of IMP dehydrogenase activity and intracellular GTP content to 25 and 36%, respectively, of the control values within 1.5 hr. The differentiation and decrease in the GTP pool induced by SM-108 were blocked by the presence of 25 μM guanine or guanosine. SM-108 also induced erythroid differentiation of K562 subline cells transfected with pMSG (K562/pMSG), which have an additional salvage pathway for GMP production from xanthine. The addition of 100 μM xanthine prevented erythroid differentiation of this subline and restored the GTP pool. These findings suggest that the induction of erythroid differentiation of K562 cells by SM-108 may be due to an early decrease in IMP dehydrogenase activity and a subsequent decrease in GTP content in the cells. Thus, purine metabolism may have an important role in SM-108-induced differentiation.

The purine antagonist 4-carbamoylimidazolium 5-olate (SM-108†) inhibits the proliferation of various kinds of neoplasms [1–4] and is now in phase I and II clinical trials [5]. SM-108 is thought to be phosphoribosylated to its nucleotide form by adenine phosphoribosyltransferase (EC 2.4.2.7) and then to inhibit inosine 5′-monophosphate dehydrogenase (EC 1.2.1.14; IMP dehydrogenase), a key enzyme in *de novo* purine synthesis [4].

Human leukemic K562 cells have been shown to undergo erythroid differentiation upon treatment with various inducers such as hemin, butyrate [6], and herbimycin A [7], and their responses to these compounds are thought to be mediated by specific receptors on the cell membrane [6]. Recently, several inhibitors of IMP dehydrogenase, tiazofurin [8, 9], ribavirin and mycophenolic acid [9], were also shown to be potent inducers of erythroid maturation of K562 leukemia cells. Cell differentiation was associated with a decrease in IMP dehydrogenase activity and a decrease in the intracellular GTP level. The addition of guanine, a precursor of GTP, inhibited the induction of K562 cell differentiation. Inhibitors of IMP dehydrogenase have also been found to induce myeloid maturation of HL-60 cells [10–14]. Moreover, we recently observed the reversal of the enzymic phenotype of thymidine metabolism during induced differentiation of leukemia cells [15, 16], and Lucas *et al.* [14] reported alterations in purine metabolism during induced maturation of leukemia cells. These findings strongly suggest that induction of differentiation may be also controlled to a critical degree by the metabolic involvement with the cell, suggesting a possible role of purine metabolism in induced differentiation.

Therefore, it was of interest to examine whether SM-108, as well as other IMP dehydrogenase inhibitors, induced erythroid differentiation of K562 leukemia cells. Furthermore, studies on the effect of SM-108 on K562 subline cells transfected with pMSG (K562/pMSG), which have an additional salvage pathway for GMP production from xanthine, should provide information on the possible role of purine metabolism in the regulation of erythroid differentiation of K562 cells.

In this paper, we report that SM-108 induced erythroid differentiation of K562 cells associated with an early decrease in the activity of IMP dehydrogenase and a subsequent decrease in the intracellular GTP concentration. We suggest that the decrease in GTP concentration may be an essential change in purine metabolism for SM-108-induced erythroid differentiation of K562 leukemia cells.

MATERIALS AND METHODS

Chemicals. [^{14}C]IMP (56 mCi/mmol) was purchased from the Amersham Corp. (Arlington Heights, IL). RPMI-1640 culture medium was from GIBCO (Grand Island, NY). SM-108 was provided by the Sumitomo Chemical Co. (Osaka, Japan). All

* Corresponding author: Dr. Shozo Irino, First Department of Internal Medicine, Kagawa Medical School, Ikenobe, Miki, Kagawa, 761-07, Japan. Tel. 0878-98-5111, Ext. 2602; FAX 0878-98-7109.

† Abbreviations: SM-108, 4-carbamoylimidazolium 5-olate; IC_{50}, drug concentration which inhibits cell growth by 50%; K562/pMSG, K562 cells transfected with pMSG; PBS, phosphate-buffered saline; and XMP, xanthine 5′-monophosphate.

other reagents were of the highest analytical grade available.

Cells and cell culture. The human chronic myelogenous leukemia cell line K562 [17] was provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan). K562/pMSG cells, which were K562 cells transfected with the shuttle vector pMSG (Pharmacia, Uppsala, Sweden) containing *E. coli* gpt (*Escherichia coli* xanthine-guanine phosphoribosyl transferase), were established in our laboratory [18]. The cells were maintained at the logarithmic phase of growth in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37° in a humidified atmosphere of 5% CO_2 in air. Cells were seeded into plastic dishes (100 × 20 mm; Becton Dickinson, Lincoln Park, NJ) at a density of 1×10^5/mL in 10 mL of medium. The doubling times of K562 and K562/pMSG cells were 24.0 and 25.6 hr, respectively. SM-108 (dissolved in RPMI-1640 culture medium) was added to the dishes at a final concentration of 50 or 100 μM and cultures were incubated for the indicated times. Cell numbers and viabilities were determined by trypan blue dye exclusion. Triplicate cultures were used in each experiment.

Hemoglobin determination. The percentage of benzidine-positive cells was scored by counts on 500 cells [19]. The solution for benzidine staining was freshly prepared before use by adding 5 μL of 30% hydrogen peroxide to 1 mL of stock solution of 0.2% benzidine/0.5% acetic acid; 50 μL of this solution was used for 50 μL volumes of suspensions of 2×10^6 cells/mL. The hemoglobin contents of cell lysates were determined from the visible absorption spectra [20]. Cell cultures were chilled on ice, and the cells were precipitated by low speed centrifugation and washed twice in ice-cold phosphate-buffered saline (PBS). They were then resuspended in 1 mL of lysing buffer [0.81% NaCl, 0.03% magnesium-acetate, 0.12% Tris (pH 7.4)] and NP-40 (Sigma Chemical Co., St. Louis, MO) was added at 0.5%. The mixture was kept on ice for 15 min, and then nuclei were precipitated by centrifugation (12,000 rpm, 40 min), and the absorption of the post-nuclear supernatant was measured at 414 nm. An optical density of 1.0 at 414 nm was assumed to correspond to a hemoglobin concentration of 0.13 mg/mL [20].

Enzyme assay. Cells were resuspended at 5×10^6 cells/mL in 0.15 M KCl, pH 7.2, and disrupted by rapid freezing in liquid nitrogen and thawing five times. The extract was centrifuged at 10,000 *g* at 4° for 10 min and the resulting supernatant was used promptly for the assay of IMP dehydrogenase activity. IMP dehydrogenase activity was measured by a reported method for radiometric assay [21] with the following slight modification: the [^{14}C]xanthine 5′-monophosphate (XMP) formed was separated from [^{14}C]IMP by cellulose TLC instead of high-voltage electrophoresis. The reaction mixture contained 100 mM Tris–HCl (pH 8.0), 100 mM KCl, 3 mM EDTA, 0.2 mM allopurinol, 0.25 mM NAD^+, 0.25 mM [^{14}C]IMP, and enzyme extract in a final volume of 25 μL. After incubation for 10 or 20 min at 37°, the reaction was stopped by boiling for 20 sec. and then the mixture was cooled promptly and centrifuged at 10,000 rpm for 10 min. Samples of 10 μL of supernatants were spotted onto cellulose TLC sheets (20 × 20 cm, Funakoshi Co., Osaka, Japan), and developed with 95% ethanol:1 M ammonium acetate solution (7:3, v/v). The ammonium acetate solution consisted of 1 M ammonium acetate and 3.3 mM EDTA adjusted to pH 5.0 with glacial acetic acid. Spots in positions corresponding to XMP and IMP were detected under UV light and cut out, and their radioactivities were measured in an Aloka, LSC-701 liquid scintillation counter. Protein was determined by the method of Lowry *et al.* [22] with bovine serum albumin as a standard. Enzyme activity was expressed as nanomoles of product formed per hour per milligram of protein.

Clonogenic assay. The drug concentration causing 50% inhibition of cell growth (IC_{50}) was determined by clonogenic assay. Cells were plated at a density of 1500 cells/mL of RPMI-1640 medium/20% fetal bovine serum/0.8% methylcellulose with various concentrations of SM-108 in plastic 35-mm Petri dishes, and after 14 days, colonies containing 50 cells or more were scored under an inverted microscope.

Analysis of ribonucleotide pools. After incubation with SM-108 for various periods, 1×10^7 cells were centrifuged for 5 min at 2000 rpm and washed twice with ice-cold PBS. The resulting cell pellets were extracted with 10% trichloroacetic acid (TCA). After vortexing, samples were centrifuged for 15 sec at 4° at 12,000 rpm, and the resulting supernatant was promptly neutralized with 0.5 M trioctylamine in freon, and analyzed by high-performance liquid chromatography (Toyo Soda, Japan) with a TSK gel DEAE-2SW column (4.6 mm i.d. × 25 cm) [23]. Samples of 10 μL were injected into the column, which was equilibrated with 0.075 M phosphate buffer (pH 7.0) containing 20% CH_3CN, and nucleotides were eluted with the same buffer at a flow rate of 1 mL/min and detected at 254 nm.

RESULTS

Effect of SM-108 on proliferation of K562 leukemia cells. Exposure of K562 cells to SM-108 resulted in a time- and concentration-dependent decrease in cellular proliferation. During 6 days of culture with 50 and 100 μM SM-108, the cell number decreased to 57 and 37%, respectively, of that in control cultures (Fig. 1). The viability of the remaining cells was more than 95% after 6 days of culture. Clonogenic assay showed that the cloning efficiency of untreated K562 cells was 61%. Treatment with various concentrations of SM-108 resulted in a concentration-dependent decrease in the total number of colonies, and the IC_{50} of SM-108 was calculated to be 35 μM (data not shown). Thus, exposure to SM-108 inhibited growth of K562 cells.

Induction of erythroid differentiation of K562 cells by SM-108. Incubation with SM-108 increased the number of benzidine-positive K562 cells. During a 6-day culture with 50 and 100 μM SM-108, 50 ± 5 and 77 ± 8%, respectively, of the cells became benzidine-positive (Fig. 2A). Hemoglobin synthesis also increased during treatment with SM-108: the

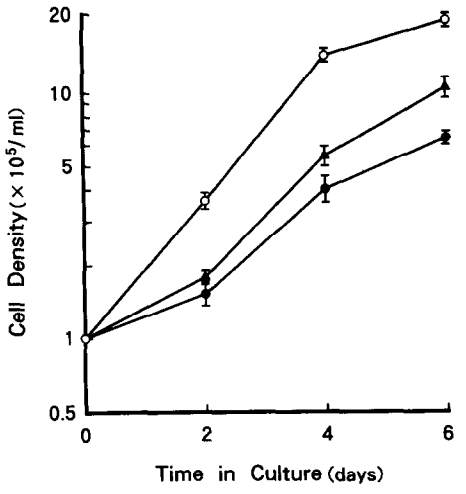

Fig. 1. Effect of SM-108 on proliferation of K562 cells. K562 cells at a concentration of 1×10^5 cells/mL were incubated with 50 and 100 μM SM-108 in triplicate cultures for 6 days. Cell viability was consistently over 95% as monitored by the trypan blue dye exclusion test. Points are means ± SEM for three determination. Key: (○) control; (▲) with 50 μM SM-108; and (●) with 100 μM SM-108.

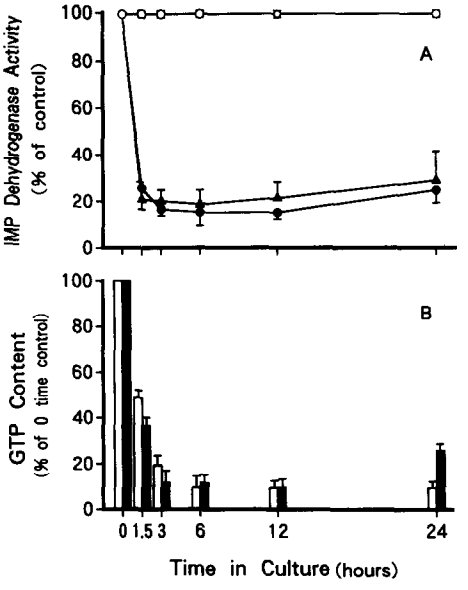

Fig. 3. Changes in IMP dehydrogenase activity and GTP concentration upon SM-108 treatment of K562 cells. Cells were incubated with or without SM-108 for various periods. The enzyme activity and GTP concentration were measured as described under Materials and Methods, and values are shown as percentages of the control values. The specific enzyme activity and GTP concentration of untreated cells at 0 time were 24.1 ± 0.3 nmol/hr/mg protein, and 4.72 ± 0.4 nmol/10^6 cells. Key: (A) Enzyme activity: (○) control; (▲) with 50 μM SM-108; and (●) with 100 μM SM-108. Points are means ± SEM for three determinations. (B) GTP concentration: open bars, with 50 μM SM-108; closed bars, with 100 μM SM-108. Columns and bars are means ± SEM for three determinations.

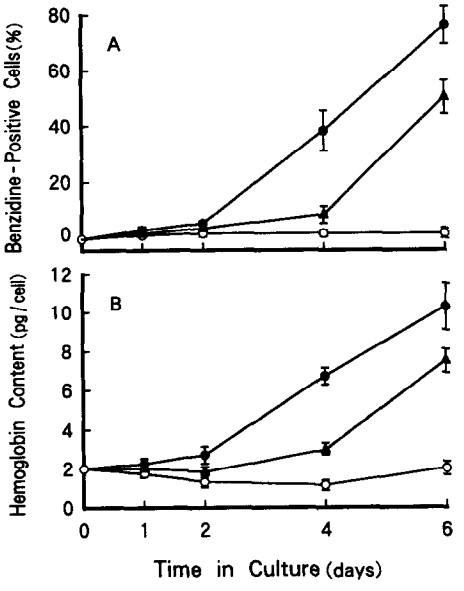

Fig. 2. Induction of erythroid differentiation of K562 cells by SM-108. The percentage of benzidine-positive cells (initial value = 0%) (A) and the intracellular hemoglobin content (initial value = 2.1 ± 0.2 pg/cell) (B) were determined as described under Materials and Methods. Points are means ± SEM for three determinations. Key: (○) control; (▲) with 50 μM SM-108; and (●) with 100 μM SM-108.

hemoglobin content was 2.1 ± 0.2 pg/cell at the time of seeding and increased to 7.7 ± 0.9 and 10.6 ± 1.3 pg/cell, during treatment with 50 and 100 μM SM-108, respectively, for the 6 days (Fig. 2B). These data indicate that SM-108 induced a time- and concentration-dependent erythroid differentiation of K562 cells.

Activity of IMP dehydrogenase and GTP concentration during differentiation of K562 cells induced by SM-108. During incubations with 50 and 100 μM SM-108, the IMP dehydrogenase activity of K562 cells decreased to 22 and 25%, respectively, of the control value within 1.5 hr, and remained low for the rest of the incubation period (Fig. 3A). Upon treatment with 50 and 100 μM SM-108, the intracellular GTP concentration also decreased to 48 and 36%, respectively, of the control level after 1.5 hr, and 20 and 12% of the control level after 3 hr and then remained low (Fig. 3B). The GTP level of untreated cells did not decrease. Incubation with SM-108 caused no significant change in the ATP pool (data not shown). The absolute values of IMP dehydrogenase activity and GTP concentration at 0 time were 24.1 ± 0.3 nmol/hr/mg protein and 4.72 nmol/10^6 cells, respectively.

Effects of purines on growth, differentiation and metabolic alterations of K562 cells. To elucidate the

Table 1. Effects of purines on growth and SM-108-induced differentiation of K562 and K562/pMSG cells

Additions	K562		K562/pMSG	
	Proliferation (%)	Benzidine-positive (%)	Proliferation (%)	Benzidine-positive (%)
None, control	100	0	100	0
SM-108, 100 μM	33	77	44	19
+ Adenine, 100 μM	106	0	119	0
+ Adenosine, 100 μM	38	73	57	16
+ Guanine, 25 μM	80	6	49	1
+ Guanine, 10 μM	52	53	ND*	ND
+ Guanosine, 25 μM	83	3	71	1
+ Guanosine, 10 μM	65	32	ND	ND

K562 and K562/pMSG cells (1×10^5 cells/mL) were incubated with 100 μM SM-108 in the absence or presence of various concentrations of purines for 6 days. Then cell numbers and benzidine-positive cells were counted. Control cultures did not contain SM-108 or purines. Control cultures of K562 and K562/pMSG cells on day 6 contained 1.8×10^6 and 1.9×10^6 cells/mL, respectively. Proliferation is expressed as a percentage of that of control cells.

* Not determined.

role of GTP depletion in induction of differentiation of K562 cells, we studied the effects of purines on differentiation of SM-108-treated K562 cells. As shown in Table 1, 100 μM adenine, but not adenosine, blocked the effect of SM-108. Upon incubation with 100 μM SM-108 for 6 days, the presence of 25 μM guanosine decreased the number of benzidine-positive cells from 77 to 3% and increased proliferation from 33 to 83% of the control. Similar extents of inhibition of erythroid differentiation and restoration of proliferation were observed with 25 μM guanine. Concentrations of 10 μM guanosine or guanine were less effective.

The effects of guanosine on IMP dehydrogenase activity and GTP concentration are shown in Table 2. Addition of 25 μM guanosine did not affect the reduction of IMP dehydrogenase activity by SM-108 alone to 13% of the control value, but prevented a decrease in the GTP pool in SM-108-treated K562 cells, the GTP level increasing from 12 to 225% of the control value.

Effect of SM-108 on K562/pMSG cells. Table 1 shows that SM-108 also induced erythroid differentiation of K562/pMSG cells. During a 6-day culture of K562/pMSG cells with 100 μM SM-108, the cell number decreased to 44% of the control, and 19% of the cells became benzidine-positive. The effects of purine bases and nucleosides on SM-108-treated K562/pMSG cells were similar to those observed with K562 cells: incubation with 25 μM guanosine decreased benzidine-positive cells from 19 to 1%, and increased proliferation from 44 to 71% of the control value. Thus, 25 μM guanosine blocked erythroid differentiation of both K562 and K562/pMSG cells.

Effects of xanthine on erythroid differentiation and altered purine metabolism of K562/pMSG cells. The effects of xanthine on erythroid differentiation of SM-108-treated K562 and K562/pMSG cells are shown in Table 2. The presence of 100 μM xanthine during incubation of K562/pMSG cells with SM-108 for 6 days reduced benzidine-positive cells from 19 to 4% and increased proliferation from 44 to 78% of the control value. In contrast, xanthine did not affect the proliferation of K562 cells or their percentage of benzidine-positive cells.

The effects of guanosine and xanthine on SM-108-induced metabolic alterations in K562/pMSG cells are also shown in Table 2. The presence of xanthine did not affect the IMP dehydrogenase activity of SM-108-treated K562/pMSG cells, but increased their GTP pool from 33 to 68% of that of control cells. The presence of 25 μM guanosine also increased the GTP content from 33 to 368% of the control level. These results show that a decrease in the GTP level, not in IMP dehydrogenase activity, is essential for erythroid differentiation of K562 cells.

DISCUSSION

The present study clearly demonstrates that SM-108 inhibited proliferation of K562 cells and induced their erythroid differentiation time and concentration dependently. As far as we know, this is the first report that SM-108 induces erythroid differentiation of K562 cells.

This erythroid differentiation was associated by marked decreases in IMP dehydrogenase activity and the intracellular GTP pool within 1.5 hr. There are reports that during erythroid differentiation of K562 cells by tiazofurin, an inhibitor of IMP dehydrogenase, both the enzyme activity and GTP level decreased [8, 9]. In the previous studies, metabolic alterations were examined after a 3-hr treatment. But we detected marked changes in purine metabolism within 1.5 hr, and observed a greater decrease in metabolic capacities than those seen previously. Our results suggested that the response of K562 cells to SM-108 is initiated very rapidly and that SM-108 is a more potent inducer of erythroid differentiation than other drugs reported previously. Our results also suggested that an early

Table 2. Effects of guanosine and xanthine on SM-108-induced differentiation and intracellular metabolic alterations and K562 and K562/pMSG cells

Cell	Additions	Proliferation (%)	Benzidine-positive (%)	GIP content (%)	IMP dehydrogenase activity(%)
K562					
	None, control	100	0	100	100
	SM-108, 100 μM	33	77	12	13
	+ Guanosine, 25 μM	83	3	225	13
	+ Xanthine, 100 μM	27	60	ND*	ND
K562/pMSG					
	None, control	100	0	100	100
	SM-108, 100 μM	44	19	33	3
	+ Guanosine, 25 μM	71	1	368	5
	+ Xanthine, 100 μM	78	4	68	4

K562 and K562/pMSG cells (1×10^5 cells/mL) were incubated with 100 μM SM-108 in the absence or presence of guanosine (25 μM) or xanthine (100 μM) for 6 days. Then proliferation and benzidine-positive cells were determined. The GTP level after incubation for 3 hr and the IMP dehydrogenase activity after 6 hr were measured. Control cultures did not contain SM-108, guanosine or xanthine. Proliferation, the GTP pool and IMP dehydrogenase activity are expressed as percentages of those of control cells. The numbers of K562 and K562/pMSG cells in control cultures on day 6 were 1.8×10^6 and 1.9×10^6 cells/mL, respectively. In control cultures of K562 and K562/pMSG cells, the GTP concentrations were 4.72 and 2.33 nmol/10^6 cells, and the IMP dehydrogenase activities were 23.8 and 24.1 nmol/hr/mg protein.

* Not determined.

decrease in IMP dehydrogenase activity and a decrease in the GTP pool induced by SM-108 may trigger erythroid differentiation, and maintenance of these factors at reduced levels may be favourable for initiation of differentiation by specific receptors on the cell membrane, as described previously [7].

Adenine, but not adenosine, reversed the growth inhibition and differentiation of K562 cells by SM-108 (Table 1). Moreover, addition of SM-108 to a cytosolic extract of K562 cells did not inhibit IMP dehydrogenase activity (data not shown), suggesting that SM-108 was not converted to its active form in cytosolic extracts. Thus, adenine may block the conversion of SM-108 to its active form competitively with adenine phosphoribosyltransferase. Guanine or guanosine should prevent depletion of the GTP pool by SM-108 through the salvage pathway from guanine to GMP. A high concentration of guanosine (100 μM) did not reverse differentiation, and guanosine itself induced erythroid differentiation of K562 cells (data not shown). However, 25 μM guanosine blocked differentiation and restored the GTP content without affecting IMP dehydrogenase activity. These observations are consistent with previous reports that guanosine reversed the induction of differentiation in K562 [8] and HL-60 [9] cells. Therefore, a decrease in the GTP content, not in IMP dehydrogenase activity, may be the essential biochemical factor for initiation of erythroid differentiation of K562 cells.

We used a subline of K562 cells, K562/pMSG, to clarify this possibility, because this subline has an additional salvage pathway for GMP production from xanthine through the enzyme xanthine-guanine phosphoribosyl transferase [18]. Results showed that SM-108 also induced erythroid differentiation of K562/pMSG cells, although it induced fewer benzidine-positive K562/pMSG cells than those of the parent K562 cells (19 vs 77%). The reason for this might be that K562/pMSG cells have xanthine-guanine phosphoribosyl transferase and may reverse the blockage of the *de novo* pathway for GTP production by SM-108. This possibility was supported by the finding that the GTP content of SM-108-treated K562/pMSG cells (33% of the control) was higher than that of K562 cells (12% of the control) (Table 2).

The addition of guanosine to SM-108-treated K562/pMSG cells prevented the decrease in the GTP pool and differentiation without restoring the IMP dehydrogenase activity, as observed with the parent K562 cells (Table 2). Xanthine did not block the differentiation of the parent cells, but it prevented differentiation in the subline cells and restored their GTP content, without affecting their decreased IMP dehydrogenase activity. Therefore, it is clear that subline K562/pMSG cells have an additional salvage pathway for GMP production from xanthine, besides the pathway from guanine. These facts indicate that the rapid decrease in the GTP content upon SM-108 treatment, regardless of the inhibition site of the *de novo* or salvage pathway, triggers induction of erythroid differentiation of K562 cells. From these data it might be expected that addition of an inhibitor of the salvage pathway for xanthine utilization for GMP production to K562/pMSG cells rescued by xanthine might induce their differentiation.

Lucas *et al.* [14] demonstrated alterations in purine metabolism in HL-60 cells during maturation. Moreover, we have reported reciprocal regulation of thymidine metabolism during growth of human leukemia-lymphoma cells in culture [24], and have also demonstrated that the inductions of differentiation of HL-60 and U937 cells resulted in reversal of the enzymic phenotype of thymidine

metabolism [15, 16]. In accordance with these observations, the present study suggests that SM-108 alters purine metabolism, resulting in erythroid differentiation of K562 cells.

We conclude that upon treatment with SM-108, the depletion of intracellular GTP following inhibition of IMP dehydrogenase may trigger differentiation of K562 cells.

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