Rapid up-regulation of P2Y messengers during granulocytic differentiation of HL-60 cells

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Abstract HL-60 cells are human promyelocytic cells expressing two ATP receptors: the $P2Y_2$ and $P2Y_{11}$ subtypes. Our Northern blotting experiments have shown that $P2Y_2$ and $P2Y_{11}$ messengers were up-regulated in these cells, rapidly and independently of protein synthesis, following treatment with granulocytic differentiating agents such as retinoic acid, dimethylsulfoxide, granulocyte-colony stimulating factor, dibutyryl cyclic AMP and ATP. AR-C67085 and adenosine 5′-*O*-(3-thiotriphosphate), two potent agonists of the recombinant $P2Y_{11}$ receptor, increased intracellular cAMP concentration in HL-60 cells more potently than ATP itself. These observations support the conclusion that the effect of ATP on HL-60 cell differentiation is mediated by the $P2Y_{11}$ receptor.

Key words: P2Y receptor; HL-60; Granulocyte; $P2Y_2$; $P2Y_{11}$

1. Introduction

HL-60 cells are an established human promyelocytic leukemia cell line. They are induced to differentiate into neutrophil-like cells by various agents including retinoic acid (RA) [1], dimethylsulfoxide (Me_2SO) [2], granulocyte-colony stimulating factor (G-CSF) [3] and cell-permeable forms of cyclic AMP (cAMP) such as N^6,2′-*O*-dibutyryl cAMP (dbcAMP) [4]. More recently, extracellular ATP has been reported to induce growth inhibition and differentiation of HL-60 cells into granulocytes through an increase in the intracellular cAMP concentration [5–7]. Phorbol 12-myristate 13-acetate (TPA) and 1α,25-dihydroxycholecalciferol (1,25-$(OH)_2D_3$) are known to differentiate these cells into monocyte-like cells [8,9].

Nucleotide receptors have been classified into the intrinsic cation channel P2X family and the G-protein-linked receptor P2Y family. Two subtypes of P2Y receptors have been described on HL-60 cells. The activation of $P2Y_2$ receptors by ATP or UTP increases the cytosolic calcium concentration [10,11] and in cells differentiated by dbcAMP enhances the superoxide anion production [12]. ATP is also able to induce an intracellular cAMP increase and the granulocytic differentiation of HL-60 cells through the activation of another P2Y subtype [5,7,13]. The recently cloned $P2Y_{11}$ receptor is dually coupled to phospholipase C and adenylyl cyclase stimulation [14]. $P2Y_{11}$ mRNA has been detected by Northern blotting in HL-60 cells [14] and the pharmacological profile of the recombinant $P2Y_{11}$ receptor closely matched that of the ATP stimulation of cAMP formation in HL-60 cells [15]. These data suggest the involvement of the $P2Y_{11}$ receptor in the granulocytic differentiation of HL-60 cells by ATP. In the present paper, we have studied the regulation of $P2Y_2$ and $P2Y_{11}$ messengers in HL-60 cells during granulocytic differentiation. Furthermore, we have investigated the effect on HL-60 cells of potent agonists of the recombinant $P2Y_{11}$ receptor.

2. Materials and methods

2.1. Materials

HL-60 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). RPMI 1640 medium, fetal bovine serum (FBS) and antibiotics were purchased from Gibco BRL (Grand Island, NY, USA). ATP, ATPγS (adenosine 5′-*O*-(3-thiotriphosphate)), cycloheximide, all-*trans*-RA, 1,25-$(OH)_2D_3$, dbcAMP, Me_2SO, TPA and G-CSF were obtained from Sigma (St. Louis, MO, USA). Rolipram was a gift from the Laboratoires Jacques Logeais (Trappes, France). AR-C67085 (2-propylthio-β,γ-dichloromethylene-D-ATP) was a generous gift of Dr. J.D. Turner and Dr. P. Leff (Astra Charnwood).

2.2. Cell culture

HL-60 cells were cultured at 37°C with 5% CO_2 in the following complete medium: 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B and 5 mM L-glutamine in RPMI 1640 medium. The HL-60 cells were differentiated by the addition of various agents during different times in the culture medium: 160 nM Me_2SO, 1 μM RA, 200 μM dbcAMP, 10 ng/ml G-CSF, 100 μM ATPγS, 100 nM TPA or 10 nM 1,25-$(OH)_2D_3$.

2.3. Northern blot analysis

HL-60 cells (10^6 cells/ml) were incubated for various times in the presence of the differentiating agents. They were then centrifuged and the pellets were stored at −80°C. The total RNA extraction was made using the RNeasy kit (Qiagen). Fifteen μg of total RNA was loaded in each lane. The $P2Y_2$ and $P2Y_{11}$ probes corresponded to a large fragment of the open reading frame of these two genes (TM1 to TM7). The blots were prehybridized for 8 h at 42°C in 50% formamide, 0.3%

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Abbreviations: ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); cAMP, cyclic AMP; FBS, fetal bovine serum; G-CSF, granulocyte-colony stimulating factor; dbcAMP, N^6,2′-*O*-dibutyryl cAMP; TPA, phorbol 12-myristate 13-acetate; RA, retinoic acid; 1,25-$(OH)_2D_3$, 1α,25-dihydroxycholecalciferol

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SDS solution and hybridized in the same solution supplemented with 10% dextran sulfate and the α-^{32}P-labeled probe. The final washing conditions were 0.2 SSC and 0.1% SDS at 65°C. The blots were exposed during 6 days and visualized using the PhosphorImager SI (Molecular Dynamics). The presence of equal amounts of RNA on each lane was checked using acridine-orange coloration.

2.4. cAMP measurements

HL-60 cells were cultured in RPMI 1640 complete medium. Cells were centrifuged, resuspended at a density of 3×10^6 cells/ml for 30 min in Krebs–Ringer HEPES buffer of the following composition (124 mM NaCl, 5 mM KCl, 1.25 mM $MgSO_4$, 1.45 mM $CaCl_2$, 1.25 mM KH_2PO_4, 25 mM HEPES (pH 7.4) and 8 mM glucose) containing 25 μM rolipram and incubated in the same medium for 15 min in the presence of the agonists. The incubation was stopped by the addition of 1 ml 0.1 M HCl. The medium was dried up, resuspended in water and diluted as required. cAMP was quantified by radioimmunoassay after acetylation as previously described [16]. EC_{50} values were obtained by curve fitting (Sigma Plot: version 2.0).

2.5. FACS analysis

HL-60 cells were incubated with the tested agents for 4 days, the medium and agents being renewed after 2 days. The cells were then centrifuged, concentrated (5×10^6 cells/ml) in phosphate-buffered saline–0.1% bovine serum albumin, containing naive mouse serum (1/25) for 30 min at room temperature, washed and resuspended at the same concentration. One hundred μl of the suspension was incubated for 30 min at room temperature, in the dark, with the phycoerythrin-conjugated IGg2A specific monoclonal antibody (1/100) directed against the surface CD11b protein (Becton Dickinson). After washing, the fluorescence of 5000 cells/tube was assayed by a FACScan flow cytofluorometer (Becton Dickinson). Data were analyzed using the CELLQuest v3.1 software (Becton Dickinson).

3. Results

We have tested the effect of several differentiating agents on the level of $P2Y_2$ and $P2Y_{11}$ messengers by Northern blotting experiments (Fig. 1). Several messengers were detected for each receptor. For the $P2Y_{11}$ receptor, an effect on the level of messengers was observed at an early stage (after 1 h) for all the tested agents: RA (1 μM), Me_2SO (1.25%), dbcAMP (200 μM), G-CSF (10 ng/ml) and ATPγS (100 μM) (Fig. 1A–C). A decrease in messenger levels was observed after this first peak (1–2 h) and a second weakly detectable increase was in general observed around 20 h. All the different $P2Y_{11}$ variants seemed to be up-regulated by the differentiating agents with a similar time course. However it was clear that Me_2SO, dbcAMP and ATPγS up-regulated mostly short $P2Y_{11}$ messengers (2 kb), whereas up-regulations of longer $P2Y_{11}$ messengers (2.5, 3.6, 4.3 and 6 kb) were more clearly observed with RA and G-CSF. The up-regulation observed with ATPγS was lower than with the other agents but significant. For the $P2Y_2$ receptor, up-regulations of four variants were observed after 1 h, except for Me_2SO and ATPγS which were unable to increase the level of $P2Y_2$ messengers (data not shown). The presence of equivalent amounts of RNA in each lane was checked using acridine-orange coloration (data not shown).

The response to the differentiating agents under our experimental conditions was checked by the measurement of O_2^- generation from HL-60 cells stimulated by fMLP and nucleotides, using the ferricytochrome *c* reduction assays. There was clearly no O_2^- generation in the non-differentiated HL-60 cells. On the contrary, Me_2SO- or RA-treated HL-60 cells showed a basal level of O_2^- generation and stimulation by fMLP, which was enhanced by ATP and UTP (data not shown). The amount of CD11b on the cell surface was also assessed (Fig. 2). An increase in CD11b protein expression

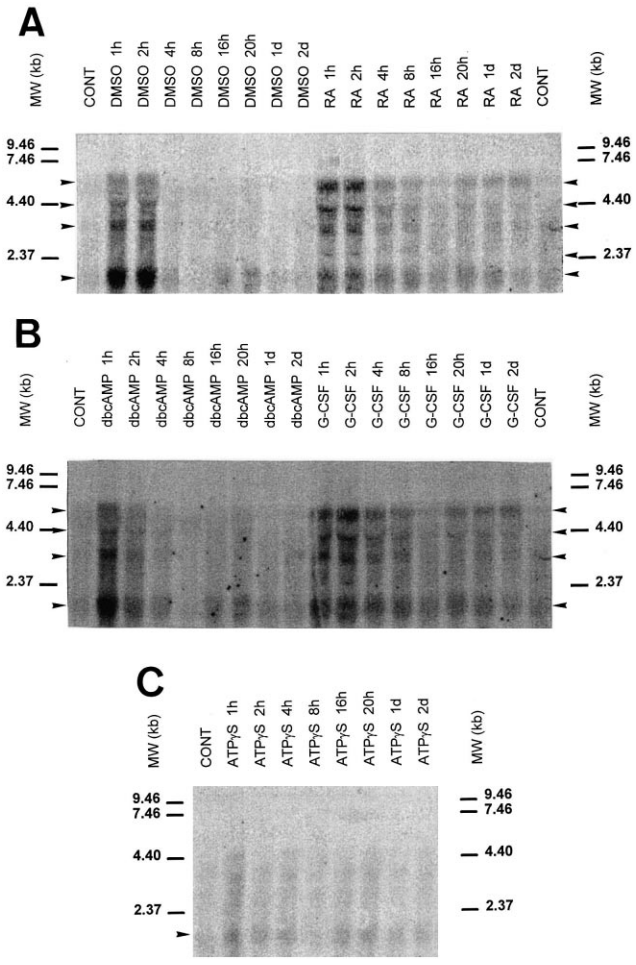

Fig. 1. Modulation of $P2Y_{11}$ messengers during granulocytic differentiation in HL-60 cells. Northern blotting experiments have been performed with total RNA (15 μg per lane) extracted from HL-60 cells exposed for different times to Me_2SO (160 nM), RA (1 μM), dbcAMP (200 μM), G-CSF (10 ng/ml) and ATPγS (100 μM). The RNA from the different cell lines were prepared with the RNeasy kit (Qiagen). The $P2Y_{11}$ probe corresponds to a large fragment of the open reading frame of the $P2Y_{11}$ gene (TM1 to TM7). The messengers are indicated by black arrowheads (2, 2.5, 3.6, 4.3 and 6 kb). The blots were treated as described in Section 2. The blots were exposed during 6 days and visualized using the PhosphorImager SI (Molecular Dynamics). These Northern blotting experiments have been reproduced twice.

was observed for all tested agents (Fig. 2A–D). Relative to RA and G-CSF alone, their combination with ATPγS enhanced the expression of CD11b (Fig. 2C,D), similar to that previously reported for the RA and G-CSF combination [17]. AR-C67085, which is a potent agonist of the $P2Y_{11}$ receptor [15], induces a significant increase (Fig. 2E) whereas UTP, which activates $P2Y_2$ receptors, did not (data not shown).

The up-regulation of $P2Y_2$ and $P2Y_{11}$ messengers at an early stage by RA was maintained, with the same time course, after cell treatment by cycloheximide (50 μg/ml) (data not shown).

TPA and 1,25-$(OH)_2D_3$, known inducers of the monocytic differentiation of HL-60 cells, were also tested in this study. TPA and 1,25-$(OH)_2D_3$ did not up-regulate $P2Y_{11}$ mRNA (data not shown). A detectable down-regulation of $P2Y_2$ transcripts was observed with TPA (data not shown).

The ATP derivative AR-C67085, which is inactive at $P2Y_1$ receptors and is an antagonist of the $P2T_{AC}$ receptor [18], was recently shown to be a potent agonist of the recombinant $P2Y_{11}$ receptor [15]. AR-C67085 increased cAMP in HL-60 cells more potently than ATP itself and with a similar potency as ATPγS (EC_{50} AR-C67085 = 32.2 ± 6.4 μM; EC_{50} ATPγS = 32.4 ± 5.8 μM; EC_{50} ATP = 282 ± 31 μM; mean ± S.D. of three independent experiments) (Fig. 3). The maximal effect of AR-C67085 was lower than that of ATP and ATPγS, as described for the recombinant $P2Y_{11}$ receptor [15].

4. Discussion

HL-60 cells are promyelocytic cells which are able to differentiate into granulocytes or monocytic cells depending on the stimuli. Pharmacological data obtained in HL-60 cells are compatible with the expression of $P2Y_2$ and $P2Y_{11}$ receptors [5,10]. Furthermore, Northern blotting has demonstrated the presence of $P2Y_2$ [19] and $P2Y_{11}$ [14] transcripts in the non-differentiated HL-60 cells. It was also previously reported that dbcAMP induces a rapid up-regulation of $P2Y_2$ mRNA in HL-60 cells [19] and that the $P2Y_{11}$ 2 kb length mRNA level

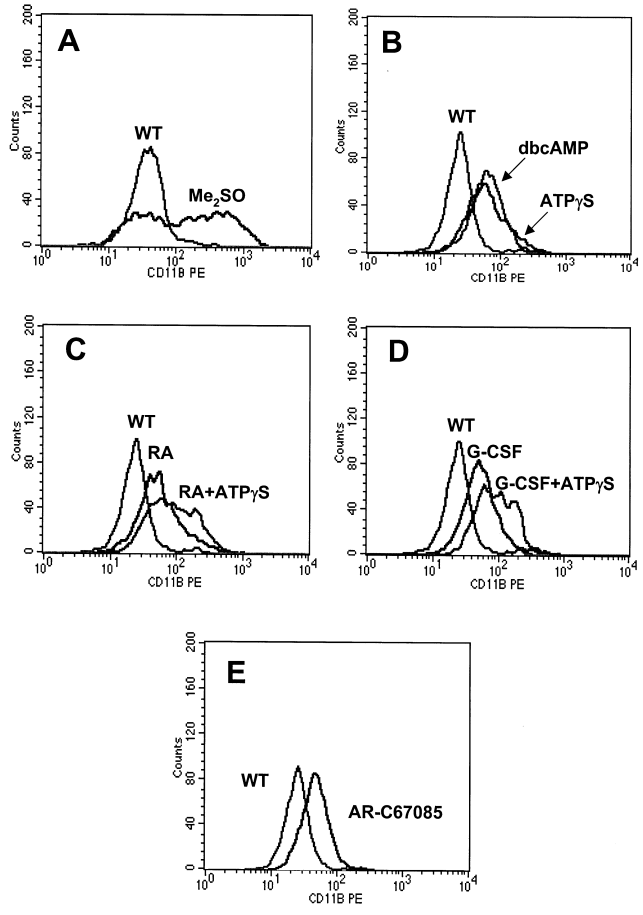

Fig. 2. FACS analysis of CD11b expression in HL-60 cells during granulocytic differentiation. HL-60 cells were exposed to Me_2SO (160 nM), RA (1 μM), dbcAMP (200 μM), G-CSF (10 nM), ATPγS (100 μM), AR-C67085 (100 μM) or a combination of two agents (RA 1 μM+ATPγS 100 μM and G-CSF 10 nM+ATPγS 100 μM) during 4 days. The cells were treated as described in Section 2. Data were obtained and analyzed using the FACScan flow cytofluorometer and the CELLQuest v3.1 software (Becton Dickinson). These data were reproduced in three independent experiments.

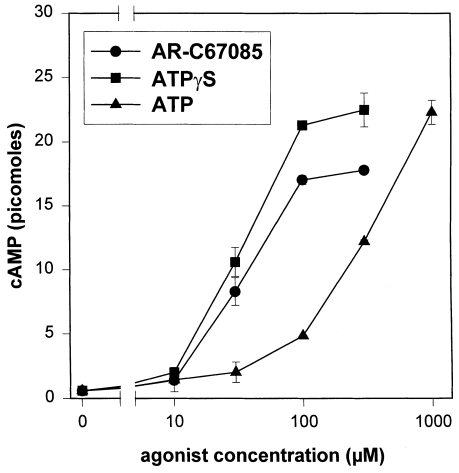

Fig. 3. Effect of AR-C67085, ATPγS and ATP on cAMP accumulation in HL-60 cells. HL-60 cells were incubated during 15 min with various concentrations of ATP, ATPγS and AR-C67085. The data are the mean ± S.D. of triplicate points obtained in one experiment representative of three.

is increased following a 6 day exposure to RA or Me_2SO [14]. From these latest results it was unclear if $P2Y_{11}$ mRNA up-regulation was a consequence of granulocytic differentiation or might constitute an early event preceding that differentiation. In the present study, we have shown that the level of $P2Y_2$ and $P2Y_{11}$ messengers was strongly increased when the cells were stimulated to differentiate into neutrophil-like cells by RA, dbcAMP and G-CSF. Me_2SO and ATPγS were only able to up-regulate $P2Y_{11}$ messengers. TPA and 1,25-$(OH)_2D_3$ did not up-regulate $P2Y_{11}$ mRNA and, as described previously, TPA down-regulated $P2Y_2$ mRNA [19].

The up-regulation of P2Y messengers was observed at an early time of stimulation by the differentiating agent (1 h) and was maintained during several hours. In some cases, a second weaker increase around 20 h of differentiation was observed. The up-regulation at an early stage by RA was not inhibited by a treatment of the cells with cycloheximide, suggesting that $P2Y_2$ and $P2Y_{11}$ receptors behave as immediate early genes of RA in the granulocytic differentiating process of the HL-60 cells. This is reminiscent of previous reports showing that the $P2Y_2$ receptor behaves as an early gene in response to glucocorticoids in activated mouse thymocytes [20]. Up-regulation of $P2Y_2$ mRNA by retinoids has also been observed in human uterine cervical cells [21].

Several messengers were detected for each receptor in our Northern blotting experiments. This has been reported previously for the $P2Y_2$ receptor [21,22]. The presence of at least one intron in the $P2Y_{11}$ open reading frame has also been previously reported [14]. Alternative splicing and the potential existence of alternative polyadenylation sites in the non-coding regions of $P2Y_2$ and $P2Y_{11}$ messengers could explain the existence of at least four variants for each subtype. Each variant seemed to follow a similar time course of regulation but some variants were more up-regulated than others depending on the agent tested. This was particularly clear on the Northern blotting data showing the regulation of $P2Y_{11}$ messengers by Me_2SO and RA. Me_2SO up-regulated strongly a short variant of the $P2Y_{11}$ messenger whereas RA up-regulated longer variants. At this stage the physiological relevance of these variants and their respective involvement in the gran-

ulocytic differentiation process of the HL-60 cells remain unclear.

The pharmacological profile of the recombinant $P2Y_{11}$ receptor [15] is similar to that of the cAMP and differentiation responses of HL-60 cells to ATP [7]. In particular, the rank order of potency of various ATP analogs was identical: ATPγS ≈ BzATP ≈ dATP > ATP. The fact that the absolute EC_{50} values do not match up could be due to the high expression of recombinant $P2Y_{11}$ receptors in CHO-K1 transfected cells. The study of the recombinant $P2Y_{11}$ receptor revealed that the ATP derivative AR-C67085 was 12-fold more potent than ATP in stimulating cAMP accumulation. Our observation that it is also more potent than ATP in increasing cAMP in HL-60 cells whereas UTP has no cAMP or differentiation effect strengthens the conclusion that these effects of ATP are mediated by the $P2Y_{11}$ receptor.

In this study we have thus demonstrated that agents inducing the granulocytic differentiation of HL-60 cells via diverse signalling pathways share the ability to rapidly up-regulate the $P2Y_{11}$ receptor, which is the ATP receptor involved in this differentiation. This suggests that ATP might have a physiological role in the differentiation induced by other mediators. In a similar way, RA has been shown to up-regulate the G-CSF receptor [17,23] and Stat1/2 [24] mRNA in HL-60 cells, but only the last effect constitutes an immediate early gene response. Targeting of the murine $P2Y_{11}$ gene is under way to determine the physiological importance of this receptor in the maturation of neutrophils.

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References

[1] Breitman, T.R., Selonick, S.E. and Collins, S.J. (1980) Proc. Natl. Acad. Sci. USA 77, 2936–2940.
[2] Collins, S.J., Ruscetti, S.W., Gallagher, R.E. and Gallo, R.C. (1978) Proc. Natl. Acad. Sci. USA 75, 2458–2462.
[3] Yamaguchi, T., Yamaguchi, T. and Hayakawa, T. (1998) Arch. Biochem. Biophys. 353, 93–100.
[4] Chaplinski, T.J. and Niedel, J.E. (1982) J. Clin. Invest. 70, 953–964.
[5] Jiang, L., Foster, F.M., Ward, P., Tasevski, V., Luttrell, B.M. and Conigrave, A.D. (1997) Biochem. Biophys. Res. Commun. 232, 626–630.
[6] Seetulsingh-Goorah, S.P. (1998) Biochem. Biophys. Res. Commun. 250, 390–396.
[7] Conigrave, A.D., Lee, J.Y., Van Der Weyden, L., Jiang, L., Ward, P., Tasevski, V., Luttrell, B.M. and Morris, M.B. (1998) Br. J. Pharmacol. 124, 1580–1585.
[8] Blaineau, C., Avner, P., Tunnacliffe, A. and Goodfellow, P. (1983) EMBO J. 11, 2007–2012.
[9] Bar-Shavit, Z., Teitelbaum, S.L., Reitsma, P., Hall, A., Pegg, L.E., Trial, J. and Khan, A.J. (1983) Proc. Natl. Acad. Sci. USA 80, 5907–5911.
[10] Dubyak, G.R., Cowen, D.S. and Meuller, L.M. (1988) J. Biol. Chem. 263, 18108–18117.
[11] Stutchfield, J. and Cockroft, S. (1990) FEBS Lett. 262, 256–258.
[12] Seifert, R. and Schultz, G. (1989) Trends Pharmacol. Sci. 10, 365–369.
[13] Choi, S.-Y. and Kim, K.-T. (1997) Biochem. Pharmacol. 53, 429–432.
[14] Communi, D., Govaerts, C., Parmentier, M. and Boeynaems, J.M. (1997) J. Biol. Chem. 272, 31969–31973.
[15] Communi, D., Robaye, B. and Boeynaems, J.M. (1999) Br. J. Pharmacol. 128, 1199–1206.
[16] Brooker, G., Harper, J.F., Teraseki, W.L. and Moylan, R.D. (1979) Adv. Cyclic Nucleotide Res. 10, 1–33.
[17] Gianni, M., Terao, M., Zanotta, S., Barbui, T., Rambaldi, A. and Garattini, E. (1994) Blood 83, 1909–1921.
[18] Humphries, R.G., Tomlinson, W., Clegg, J.A., Ingall, A.H., Kindon, N.D. and Leff, P. (1995) Br. J. Pharmacol. 115, 1110–1116.
[19] Martin, K.A., Kertesy, S.B. and Dubyak, G.R. (1997) Mol. Pharmacol. 51, 97–108.
[20] Koshiba, M., Apasov, S., Sverdlov, V., Chen, P., Erb, L., Turner, J.T., Weisman, G.A. and Sitkovsky, M.V. (1997) Proc. Natl. Acad. Sci. USA 94, 831–836.
[21] Gorodeski, G.I., Burfeind, P., Gan, S.U., Pal, D. and Abdul-Karim, F.W. (1998) Am. J. Physiol. 275, C758–C765.
[22] Parr, C.E., Sullivan, D.M., Paradiso, A.M., Lazarowski, E.R., Burch, L.H., Olsen, J.C., Erb, L., Weisman, G.A., Boucher, R.C. and Turner, J.T. (1994) Proc. Natl. Acad. Sci. USA 91, 3275–3279.
[23] Tkatch, L.S., Rubin, K.A., Ziegler, S.F. and Tweardy, D.J. (1995) J. Leuk. Biol. 57, 964–971.
[24] Gianni, M., Terao, M., Fortino, I., LiCalzi, M., Viggiano, V., Barbui, T., Rambaldi, A. and Garattini, E. (1997) Blood 89, 1001–1012.