Sodium nitroprusside inhibits proliferation and putrescine synthesis in human colon carcinoma cells

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Abstract In human colon carcinoma HT-29 $Glc^{-/+}$ cells, L-arginine is the common precursor of polyamines which are absolutely necessary for cellular proliferation and nitric oxide (NO) with reported anti-proliferative activity. The aim of the present work was to test the effect of the NO donor sodium nitroprusside (SNP) on polyamine synthesis and cellular growth in HT-29 cells. SNP in the micromolar range inhibits cellular putrescine synthesis and this effect is greatly reversed by haemoglobin, supporting the view that the effect of SNP is related to the generation of NO. This corresponds to the inhibition by SNP of ornithine decarboxylase activity. Furthermore, SNP inhibits cellular proliferation. The effect of SNP is reversed by haemoglobin after 2 days of treatment but not after 4 days. Although no acute toxic effect of SNP was detected after 90 min incubation, it greatly enhanced the cellular death rate after several days in culture as estimated by the LDH leakage test. In conclusion, our data raise the possibility of an inhibitory interrelationship between NO and polyamine metabolic pathways. NO induced inhibition of putrescine synthesis and growth in HT-29 cells is discussed from a causal perspective.

Key words: Sodium nitroprusside; Nitric oxide; Polyamine; Colon carcinoma cell

1. Introduction

Since normal enterocytes isolated from the small intestine of mammals do not survive for more than 1 h [1], intestinal cell proliferation and differentiation may be analysed in malignant cells including HT-29 cells originating from a human colon carcinoma [2]. Differentiated HT-29 Glc^- cells are selected in a hexose-free culture medium [3]. In the present work, we used Glc^- HT-29 cells which have been switched back in a D-glucose-containing medium [4]. These cells, called $Glc^{-/+}$ HT-29 cells, can spontaneously differentiate after confluency in enterocyte-like cells with an apical brush border membrane equipped with villin and dipeptidylpeptidase IV [4,5]. Polyamines are involved in the proliferation/differentiation process [6]. In HT-29 cells, these polycations can be taken up from an extracellular medium or can be generated from amino acid precursors, mainly L-arginine [7]. L-Arginine is degraded into L-ornithine and urea by arginase. L-Ornithine is the precursor of putrescine, spermidine and spermine by the action of ornithine decarboxylase, spermidine synthase and spermine synthase respectively. In HT-29 cells, the inhibition of cellular synthesis of polyamines leads to the almost complete arrest of cellular proliferation [8]. In HT-29 cells, a small part of L-arginine taken up is degraded into nitric oxide and L-citrulline [7]. NO is generally considered as anti-proliferative [9–16]. The fact that L-arginine is a precursor of metabolites with opposite effects on the cellular state led us to assess the effect of the NO donor SNP on polyamine synthesis and cellular growth and differentiation.

2. Materials and methods

2.1. Chemicals

All the ^{14}C-labelled molecules were purchased from Amersham or New England Nuclear. Gly-Pro *p*-nitroanilide, *p*-nitroaniline, sodium nitroprusside, bovine haemoglobin and putrescine dihydrochloride were from Sigma Chemicals.

2.2. HT-29 cell culture and incubation

HT-29 $Glc^{-/+}$ cells used in this study were kindly provided by A. Servin (Faculté de Pharmacie, Châtenay-Malabry, France) and cultured in standard DMEM medium containing 10% foetal calf serum, 25 mM D-glucose, 100 U/ml penicillin and 100 μg/ml streptomycin exactly as described [7]. HT-29 $Glc^{-/+}$ cells were used between passages 62 and 77 (1 passage every 7 days) and were seeded at a density of 0.40×10^6 cells per 25 cm^2 on day 0. The culture medium with or without the tested agents was changed every day and cells were isolated with EDTA-trypsin phosphate-buffered saline [7] and counted with a haemacytometer. The cells were washed and incubated in Krebs-Henseleit bicarbonate-buffered medium (pH 7.4) saturated with a mixture of O_2-CO_2 (19:1, v/v) containing 10 mM HEPES, 1.3 mM $CaCl_2$, 2 mM $MgCl_2$ and enriched with 10 mg/ml bovine serum albumin. Radioactive urea, putrescine and spermidine were separated by HPLC using a C18 partisphere column (Whatman) as described elsewhere [17]. Incorporation of L-[U-^{14}C]leucine into proteins was measured after precipitation of proteins with 10% trichloroacetic acid. Oxidation of L-[U-^{14}C]glutamine was determined by measuring $^{14}CO_2$ as described previously [17]. Nitrite was assayed using Griess reagent [18]. Basal cell content in nitrite represented 172 ± 39 pmol/10^6 cells at the end of incubation ($n = 5$).

2.3. Enzyme activities

Dipeptidylpeptidase IV was assayed using the method of Nagatsu [19] in sonicated cells recovered from the flask with a cell scraper. The assay medium was a 71 mM glycine/NaOH buffer, pH 8.7 containing 1 mM phenylmethylsulfonyl fluoride. Ornithine decarboxylase activity was determined in sonicated cells by decarboxylation of L-[1-^{14}C]ornithine as described [17]. Lactate dehydrogenase (LDH) leakage test was performed by measuring the activity of this cytosolic enzyme in the culture medium or in the adhering cells as described elsewhere [1].

2.4. Presentation of results

The results were expressed as the mean (±S.E.M.) together with the number of individual experiments performed with HT-29 cells isolated at different passages (n). The production of radioactive metabolites from precursors was calculated by reference to the specific activity of the precursors in the incubation medium. The statistical significance of differences between mean values was assessed by Student's *t*-test.

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3. Results

3.1. Nitrite synthesis from SNP

When 10 μM SNP was added for 90 min at 37°C in the incubation medium, 0.35 ± 0.09 μM nitrite ($n = 5$) was measured. When the cells were incubated in the presence of 10 μM SNP, the production of nitrite from SNP averaged 128 ± 30 pmol/10^6 cells per 90 min ($n = 5$).

3.2. Effect of SNP on polyamine synthesis

SNP (10 μM) inhibited the synthesis of radioactive putrescine from 0.1 mM L-[U-^{14}C]ornithine (Fig. 1). In the presence of both SNP (10 μM) and haemoglobin (30 μM), the inhibitory effect of SNP was partly reversed. Haemoglobin alone has no effect on putrescine synthesis. The production of spermidine from L-ornithine was also affected by SNP (10 μM). Indeed, basal spermidine production (i.e. 47 ± 15 pmol/10^6 cells per 90 min, $n = 4$) was slightly but significantly reduced (25 ± 7% inhibition, $n = 4$, $p < 0.05$ vs. basal). When SNP was used at a concentration equal to 1 μM, the inhibitory effect on putrescine production was equal to 18 ± 4% ($n = 3$, $p < 0.05$ vs. basal). The inhibition of the flux of L-ornithine through ornithine decarboxylase in isolated cells corresponded to an inhibitory effect of SNP on ODC activity (Fig. 2).

The basal production of radioactive urea from 0.4 mM L-[*guanido*-^{14}C]arginine by HT-29 cells was not affected by 10 μM SNP indicating that SNP did not affect the flux of L-arginine through arginase (data not shown).

3.3. Effect of SNP on cellular growth, metabolism, viability and differentiation

The effect of SNP on cellular growth was then assessed (Fig. 3). SNP induced in a dose-dependent manner a decrease in the number of HT-29 cells recovered in the flask. The effect of haemoglobin on SNP induced inhibition of cellular growth was investigated by adding haemoglobin at the same time as SNP. On day 3, haemoglobin reversed the effect of SNP (Fig. 4). It should be noted that haemoglobin alone significantly inhibited HT-29 cell growth.

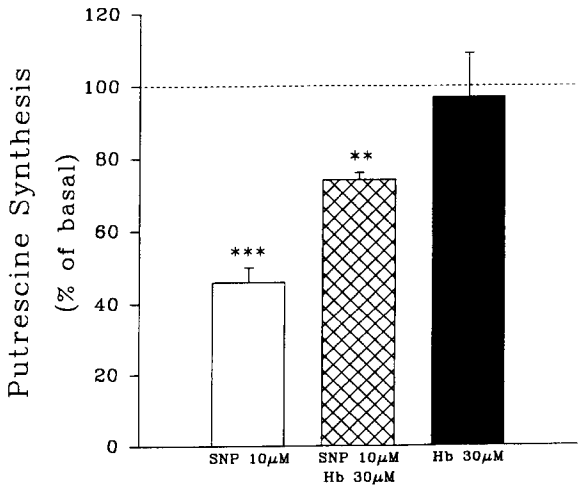

Fig. 1. Effect of SNP and haemoglobin on putrescine production from 0.1 mM L-[U-^{14}C]ornithine by HT-29 cells. HT-29 cells were isolated 5 days after seeding. Results are expressed as mean value (± S.E.M.) and represent 4 individual experiments. Basal putrescine production was equal to 1607 ± 559 pmol/10^6 cells per 90 min. $^{**}p < 0.025$ vs. SNP alone, $^{***}p < 0.001$ vs. basal.

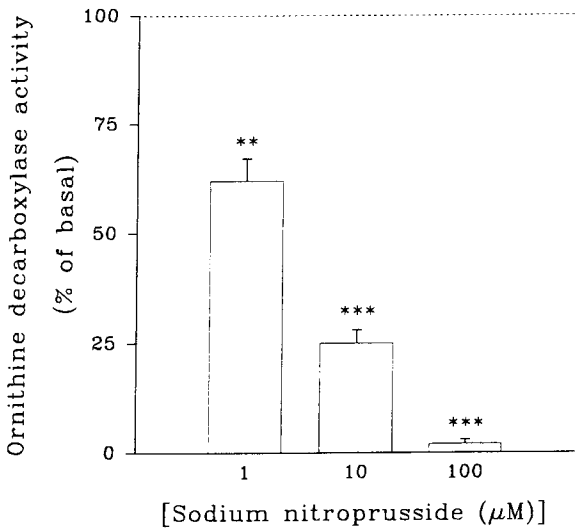

Fig. 2. Effect of SNP on ODC activity. Homogenates of HT-29 cells recovered 5–6 days after seeding were incubated in the presence of 200 μM L-[1-^{14}C]ornithine and increasing concentrations of SNP. The basal ODC activity represented 200 ± 71 pmol/10^6 cells per h. Results are expressed as mean value (± S.E.M.) and represent 5–8 individual experiments. $^{**}p < 0.005$ vs. basal, $^{***}p < 0.001$ vs. basal.

However, on day 5, haemoglobin was unable to reverse significantly the effect of 10 μM SNP. Hence, the number of cells recovered in the presence of 30 μM haemoglobin was equal to 4.67 ± 1.21 10^6 cells per flask. In the presence of 10 μM SNP or 10 μM SNP with 30 μM haemoglobin, the number of cells was equal to 0.27 ± 0.05 and 0.84 ± 0.54 10^6 per flask, respectively ($n = 3$). Furthermore, when putrescine was added at a concentration of 10 μM with 10 μM SNP, it did not reverse the effect of SNP either on days 3, 5 or 7. Thus, the number of cells recovered per flask in the presence of putrescine and SNP was 0.14 ± 0.06, 0.16 ± 0.04 and 0.06 ± 0.01 10^6 on days 3, 5 and 7, respectively. The number of cells recovered in the sole presence of 10 μM SNP was 0.16 ± 0.08, 0.18 ± 0.06 and 0.12 ± 0.05 10^6 per flask ($n = 3$ in all cases).

The effect of SNP on cell death rate was estimated by the LDH leakage test (Table 1). It should be pointed out that on day 3 and in the absence of SNP, the percentage of LDH

Table 1
Effect of SNP on cell death rate

SNP (μM)	Percentage of dead cells		
	Day 3	Day 5	Day 7
0	23 ± 8 (3)	3 ± 1 (4)	3 ± 1 (4)
0.1	ND	5 ± 1 (4)	4 ± 1 (4)
1.0	ND	52 ± 15 (4)	27 ± 11 (3)
10.0	58 ± 9 (4)	83 ± 4 (4)	72 ± 8 (4)

Percentage of dead cells was estimated by calculating the ratio of LDH activity in the culture medium as a percentage of total LDH activity (i.e. the sum of LDH activity in culture medium and adhering cells). ND, not determined. LDH total activity in nmol/min per flask = day 3, basal (no SNP) = 1205 ± 316, 10 μM SNP = 973 ± 184; day 5, basal = 5417 ± 650, 0.1 μM SNP = 3672 ± 615, 1 μM SNP = 892 ± 233, 10 μM SNP = 580 ± 306; day 7, basal = 12396 ± 745, 0.1 μM SNP = 7733 ± 898, 1.0 μM SNP 1986 ± 815, 10 μM SNP = 250 ± 85.

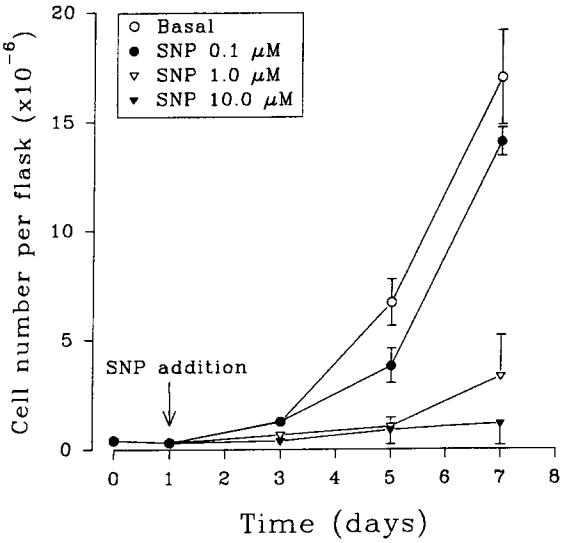

Fig. 3. Effect of SNP on HT29 cell growth. SNP was added 1 day after seeding and the culture medium with or without SNP was changed every day. Cells were isolated with trypsin and counted at different periods of time. Results are expressed as mean values (±S.E.M.) and represent 512 individual experiments.

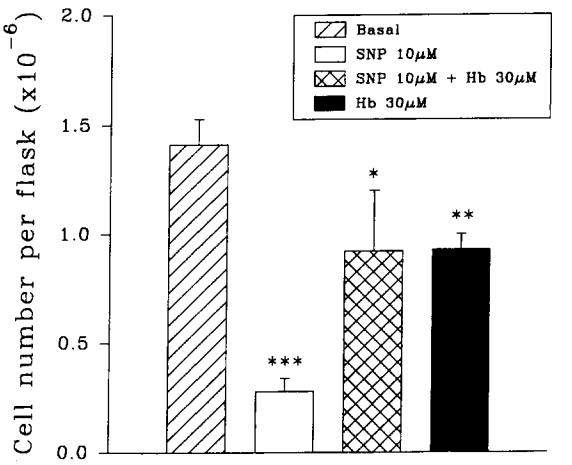

Fig. 4. Effect of SNP and haemoglobin on HT129 cells growth. SNP and haemoglobin were added together 1 day after seeding and the culture medium with or without the agents tested was changed every day. The cells were isolated and counted 3 days after seeding. Results are expressed as mean values (±S.E.M.) and represent 5 individual experiments; $^{*}p < 0.05$ vs. SNP alone, $^{**}p < 0.025$ vs. basal, $^{***}p < 0.001$ vs. basal.

recovered in the culture medium was relatively high, indicating cell death in the early days of culture after treatment with trypsin and seeding. It was found that 1 μM or more of SNP seriously affected the viability of HT-29 cells. It should noted that the remaining adhering cells were apparently not greatly affected by SNP in term of viability. Thus, the basal cellular viability on days 3, 5 or 7 as measured by the trypan blue exclusion test (i.e. 84 ± 2%, $n = 11$), remained unchanged in the presence of 1 μM SNP (84 ± 3%, $n = 8$) and was slightly reduced in the presence of 10 μM SNP (69 ± 6, $n = 11$, $p < 0.05$ vs. basal).

The effect of SNP was then tested on different metabolism and viability parameters on short-term cellular incubation (Table 2). It was observed that 10 μM SNP exerted no effect on the 3 parameters tested. The treatment with increasing concentrations of SNP did not lead to any significant increase in the activity of dipeptidyl peptidase IV (Table 3).

4. Discussion

Our data demonstrate that putrescine synthesis in isolated HT-29 cells can be strongly inhibited by 10 μM SNP while 1 μM exerts a weak inhibitory effect.

This effect was reversed by the addition of haemoglobin which suggests that SNP acts as a NO donor. Indeed, the affinity of haemoglobin for NO is very high [20]. Under our incubation conditions, and in the absence of cells, 10 μM SNP released 0.35 μM nitrite. This would correspond to the spontaneous release of NO from SNP since it has been shown that in aerobic aqueous solutions, the primary degradation product of NO is nitrite [21]. It is worth noting that HT-29 cells are able to produce nitrite from SNP. This would correspond to the cellular generation of NO from SNP as recently demonstrated in rat liver microsomes [22]. Thus, NO produced spontaneously or generated from SNP by HT-29 cells would inhibit putrescine synthesis. This inhibition is apparently due to that of ornithine decarboxylase (ODC) activity as demonstrated in the present study. ODC is a proto-oncogene which is a key regulator for cell growth and malignant transformation [23]. Polyamines modulate the transcription of growth associated genes in human colon carcinoma cells [24] and putrescine is involved in DNA, RNA and protein synthesis in intestinal epithelial cells [25].

This led us to test the effect of SNP on HT-29 cell proliferation. The inhibitory effect was marked for a concentration equal to 1 μM and total at 10 μM. The effect could be reversed by haemoglobin after 2 days of treatment, but not after 4 days indicating that the effect of SNP was due to NO production in the first case. In contrast, the effect of SNP after 4 days of culture does not seem to be primarily due to NO synthesis. Although the influence of SNP on cell growth is not a mere short-term toxic effect as measured from viability or metabolism parameters such as protein synthesis or glutamine oxidation, SNP from concentrations equal to 1 μM acts on cellular viability after several days in culture.

It may therefore be asked whether inhibition of putrescine synthesis in HT-29 cells by SNP plays a role in the decrease of cell viability and growth. The fact that putrescine added to the

Table 2
Effect of SNP on cell metabolism and viability

	Basal	SNP (10 μM)
Incorporation of 1 mM L-[U-^{14}C]leucine into proteins (nmol/10^6 cells per 90 min)	2.34 ± 0.96 (3)	2.51 ± 1.11 (3)
Oxidation of 2 mM L-[U-^{14}C]glutamine (nmol/10^6 cells per 90 min)	4.69 ± 1.30 (3)	4.93 ± 0.66 (3)
Trypan blue viabilityafter 90 min incubation (% viable cells)	93 ± 2 (3)	94 ± 1 (3)

Cells were isolated 5 days after seeding.

Table 3
Effect of SNP on HT-29 dipeptidyl peptidase IV activity

SNP (μM)	Day 3	Day 5	Day 7
0	12.2 ± 1.1 (4)	14.3 ± 1.7 (4)	20.5 ± 3.3 (4)
0.1	14.5 ± 0.3 (3)	17.0 ± 2.1 (3)	23.6 ± 4.5 (3)
1.0	15.8 ± 2.3 (3)	13.3 ± 2.1 (4)	14.7 ± 0.1 (3)
10.0	21.1 ± 4.0 (4)	ND	22.0 ± 3.7 (3)

ND, not determined. DPP IV activity was measured in adhering cells recovered with a cell scraper. Results are expressed as mU/mg protein (1 mU=1 nmol/min).

culture medium does not reverse the SNP effect suggests that although inhibition of putrescine synthesis by NO could play a role in the inhibitory effect on growth after 2 days of culture, this would not be the primary target. Hence, when an ODC inhibitor, namely difluoromethylornithine, was added to the culture medium of HT-29 cells, it inhibited cell growth, an effect which could be reversed by adding putrescine [8].

Our data show an extreme sensitivity of HT-29 cells to SNP. In other investigations, millimolar concentrations of SNP have been used to alter significantly cell proliferation [26–28]. SNP-induced cytotoxicity has been shown to be due to NO release in hepatocytes [29]. SNP has been used as a differentiating agent in several cell types [28,30]. However, in the present study, SNP did not alter the activity of the differentiation marker dipeptidylpeptidase IV. In conclusion, our data support the idea of a regulation of polyamine synthesis by NO in HT-29 cells, suggesting the possibility of interference between two metabolic pathways derived from the common precursor L-arginine. NO synthase activity is low in HT-29 cells [7] but recent data suggest that the production of NO increases in HT-29 cells by a combination of cytokines [31]. Further studies are required to determine the effect of cellular NO overproduction on cell growth.

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References

[1] Darcy-Vrillon, B., Posho, L., Morel, M.T., Bernard, F., Blachier, F., Meslin, J.C. and Duée, P.H. (1994) Pediatr. Res. 36, 175–181.
[2] Zweibaum, A., Laburthe, M., Grasset, E. and Louvard, D. (1991) in: The Gastrointestinal System IV (Field, M. and Frizzell, R.A. eds.) pp. 223–255, American Physiological Society, Washington, DC.
[3] Zweibaum, A., Pinto, M., Chevalier, G., Dussaulx, E., Triadou, N., Lacroix, B., Haffen, K., Brun, J.L. and Rousset, M. (1985) J. Cell. Physiol. 122, 21–29.
[4] Lesuffleur, T., Kornowski, A., Augeron, C., Dussaulx, E., Barbat, A., Laboisse, C. and Zweibaum, A. (1991) Int. J. Cancer 49, 731–737.
[5] Darmoul, D., Lacasa, M., Baricault, L., Marguet, D., Sapin, C., Trotot, P., Barbat, A. and Trugnan, G. (1992) J. Biol. Chem. 267, 4824–4833.
[6] Pegg, A.E. and McCann, P.P. (1982) Am. J. Physiol. 243, C212–C221.
[7] Blachier, F., Selamnia, M., Robert, V., M'Rabet-Touil, H. and Duée, P.H. (1995) Biochim. Biophys. Acta 1268, 255–262.
[8] Gamet, L., Cazenave, Y., Trocheris, V., Denis-Plouxviel, C. and Murat, J.C. (1991) Int. J. Cancer 47, 633–638.
[9] Hibbs, J.B., Taintor, R.R., Vavrin, Z. and Rachlin, E.M. (1988) Biochem. Biophys. Res. Commun. 157, 87–94.
[10] Garg, U.C. and Hassid, A. (1990) Biochem. Biophys. Res. Commun. 171, 474–479.
[11] Lepoivre, M., Chenais, B., Yapo, A., Lemaire, G., Thelander, L. and Tenu, J.P. (1990) J. Biol. Chem. 265, 14143–14149.
[12] Nguyen, T., Brunson, D., Crespi, C.L., Penman, B.W., Wishnok, J.S. and Tannenbaum, S.R. (1992) Proc. Natl. Acad. Sci. USA 89, 3030–3034.
[13] Goureau, O., Lepoivre, M., Becquet, F. and Courtois, Y. (1993) Proc. Natl. Acad. Sci. USA 90, 4276–4280.
[14] Huot, A.E., Moore, A.L., Roberts, J.D. and Hacker, M.P. (1993) Immunol. Invest. 22, 319–327.
[15] Firnhaber, C. and Murphy, M.E. (1993) Am. J. Physiol. 265, R518–R523.
[16] Ionnidis, I. and De Groot, H. (1993) Biochem. J. 296, 341–345.
[17] Blachier, F., M'Rabet-Touil, H., Posho, L., Morel, M.T., Bernard, F., Darcy-Vrillon, B. and Duée, P.H. (1992) Biochim. Biophys. Acta 1175, 21–26.
[18] Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S. and Tannenbaum, S.R. (1982) Anal. Biochem. 126, 131–138.
[19] Nagatsu, T., Hino, M., Fuyamada, H., Hayakawa, T., Sakakibara, S., Nakagawa, Y. and Takemoto, T. (1976) Anal. Biochem. 74, 466–476.
[20] Ignarro, L.J., Byrns, R.E., Buga, G.M. and Wood, K.S. (1987) Circ. Res. 61, 866–879.
[21] Ignarro, L.J., Fukuto, J.M., Griscavage, J.M., Rogers, N.E. and Byrns, R.E. (1993) Proc. Natl. Acad. Sci. USA 90, 8103–8107.
[22] Ramakrishna Rao, D.N. and Cederbaum, A.I. (1995) Arch. Biochem. Biophys. 321, 363–371.
[23] Auvinen, M., Paasinen, A., Anderson, R.C. and Hölttä, E. (1992) Nature 360, 355–358.
[24] Celano, P., Baylin, S.B. and Casero, R.A. (1989) J. Biol. Chem. 264, 8922–8927.
[25] Ginty, D.D., Osborne, D.L. and Seidel, E.R. (1989) Am. J. Physiol. 257, G145–G150.
[26] Yang, W., Ando, J., Korenaga, R., Toyo-Oka, T. and Kamiya, A. (1994) Biochem. Biophys. Res. Commun. 203, 1160–1167.
[27] Soo Lee, Y. and Wurster, R.D. (1994) Cancer Lett. 78, 19–23.
[28] Shami, P.J., Moore, J.O., Gockerman, J.P., Hathorn, J.W., Misukonis, M.A. and Weinberg, J.B. (1995) Leuk. Res. 8, 527–533.
[29] Niknahad, H. and O'Brien, P.J. (1996) Biochem. Pharmacol. 51, 1031–1039.
[30] Boss, G.R. (1989) Proc. Natl. Acad. Sci. USA 86, 7174–7178.
[31] Kolios, G., Brown, Z., Robson, R.L., Robertson, D.A.F. and Westwick, J. (1995) Br. J. Pharmacol. 116, 2866–2872.