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Clinical report

Phosphodiesterase 3 as a potential target for therapy of malignant tumors in the submandibular gland

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Phosphodiesterase (PDE) 3s have been characterized in human neoplastic submandibular gland intercalated duct HSG cells. There have been no reports on PDE3 in malignant salivary gland cells. PDE3 activity was detected in homogenates of HSG cells. About 75% of PDE3 activity in HSG cells was recovered in supernatant fractions and 25% in particulate fractions. PDE3A and 3B mRNAs were detected by reverse transcription-polymerase chain reaction in RNA from HSG cells. The nucleotide sequences of the fragments were identical to those of human PDE3A and 3B. The PDE3-specific inhibitor, cilostamide, inhibited the growth of HSG cells. Our results indicate that PDE3s may be important in the growth of HSG cells. PDE3 thus appears to be a potential new target for antiproliferative therapies. [© 2001 Lippincott Williams & Wilkins.]

Key words: Phosphodiesterase, phosphodiesterase inhibitor, salivary gland tumor.

Introduction

Eleven cyclic nucleotide phosphodiesterase gene families (PDE1–11) have been identified in various tissues or cells and characterized on the bases on their primary amino acid sequences, affinities for cAMP and cGMP, sensitivities to specific inhibitors, biochemical and physical properties, and biological regulatory mechanisms.[1–11] PDE3 forms are found in a variety of tissues, including myocardium, platelets and adipose tissue.[1–3] They are characterized by high affinities for both cAMP and cGMP, with K_m values in the range of 0.1–0.8 μM and V_{max} for cAMP 4–10 times higher than that for cGMP, and competitive inhibition of its cAMP hydrolytic activity by cGMP, cilostamide and certain positive inotropic agents, including enoximone and milrinone.[1–3] Studies with specific PDE3 inhibitors suggest that PDE3s are important in the regulation of cAMP-modulated processes, including myocardial contractility, platelet aggregation and antilipolytic action.[1–3] cDNAs encoding two distinct but related PDE3 isoforms (PDE3A and 3B) have been cloned from rat and human adipose and cardiac cDNA and genomic libraries.[1–5,12] Their deduced sequences indicate that the C-terminal regions of PDE3s contain the catalytic domain conserved among all mammalian PDEs, and that rat and human PDE3A (or 3B) are more similar than PDE3A and 3B from the same species.[1–5,12]

There are a few reports of expression of PDE3 mRNAs in tumor cells.[13–15] In a previous study we were the first to report that human PDE3A and 3B mRNAs were expressed in single-cell populations, i.e. two hepatocellular carcinoma cell lines.[16] However, no data are available as to the expression of PDE3 isoforms in submandibular gland malignant tumor cells or the role of PDE3s in these cells. Furthermore, as discussed by McGurk *et al.* the response rates of chemotherapy on salivary malignant tumors are very low and only 10% of patients have been judged to have a complete response.[17] In this report, we demonstrate, for the first time, the presence of PDE3 in human submandibular gland intercalated HSG cells. Cilostamide, a specific PDE3 inhibitor, inhibits proliferation of these cells, suggesting in important regulatory role for PDE3.

Materials and methods

Cell line

Human neoplastic submandibular gland intercalated duct HSG cells[18] were maintained in Dulbecco's

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modified Eagle medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin (Life Technologies, Grand Island, NY). Media was changed 3 times per week.

PDE activity in cell extracts

HSG cells were seeded at 1×10^6 cells/25 cm^2 flask (Nunc, Roskilde, Denmark). After 3 days, cells were washed twice with phosphate-buffered saline (PBS), harvested with a rubber policeman, homogenized in 2 ml of ice-cold homogenization buffer (100 mM TES, pH 7.4, 10 μg/ml each of pepstain, leupepsin and aprotinin, 1 mM benzamidine, 0.5 mM pefabloc, 1 mM EDTA, 0.1 mM EGTA, 5 mM $MgSO_4$, 10% glycerol) and centrifuged at 100 000 *g* for 45 min at 4°C to obtain crude particulate and supernatant fractions. Particulate fractions were suspended in 1 ml of homogenization buffer.

cAMP PDE assay

cAMP PDE activity was assayed by a modification of a previously described procedure.[19] Samples were incubated at 30°C for 10 min in a total volume (0.3 ml) containing 50 mM HEPES, pH 7.4, 0.1 mM EGTA, 8.3 mM $MgCl_2$ and 0.1 μM [^{3}H]cAMP (18 000 c.p.m.). PDE3 activity (pmol cAMP hydrolyzed/min) was measured as the cAMP PDE activity inhibited by 0.5 μM cilostamide, a specific inhibitor of PDE3, and 5 μM cGMP, a competitive inhibitor of cAMP hydrolysis by PDE3. PDE4 activity was measured as the cAMP PDE activity inhibited by 10 μM rolipram, a specific inhibitor of PDE4.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from tumor cells. (QuickPrep Total RNA extraction kit; Amersham Pharmacia Biotech, Piscataway, NJ). First-strand cDNA was generated from total RNA using the Superscript preamplification system (Life Technologies Grand Island, NY). Two specific oligonucleotide primer sets were synthesized[1,2,9,12] (Table 1). PCR amplification was carried out in a total volume of 50 μl containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, 2.5 U AmpliTag Gold (Perkin Elmer Applied Biosystems, Foster City, CA), and 0.8 μM sense and antisense primers. The PCR reaction was performed at 95°C for 1 min, 55°C for 1 min and 72°C for 1 min, for 35 cycles. Products were subjected to electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. No fragments were amplified in the absence of reverse transcriptase or RNA.

Table 1. Primer used for RT-PCR from HSG RNA

cDNA	Primer
Human PDE3A	
sense primer	5′-TCACCTCTCCAAGGGACTCCT
antisense primer	5′-CAGCATGTAAAACATCAGTGGC
Human PDE3B	
sense primer	5′-AATTCTTCCAACCATCGACC
antisense primer	5′-GCTTGTAGCACATCTGTGGC

Growth experiments

HSG cells were cultured with different concentrations of cilostamide, after which the cells were detached from the culture dish by incubating with a trypsin–EDTA solution (0.05% trypsin and 0.02% EDTA). Cell number was determined using a hemocytometer and cell viability by the Trypan blue exclusion method.

Results

PDE3 and PDE4 activities (assayed with 0.1 μM [^{3}H]cAMP as substrate) were measured. As shown in Figure 1, in extracts of HSG cells, PDE activity was inhibited by cilostamide, cGMP and rolipram, indicating the presence of both PDE3 and PDE4 enzymes.

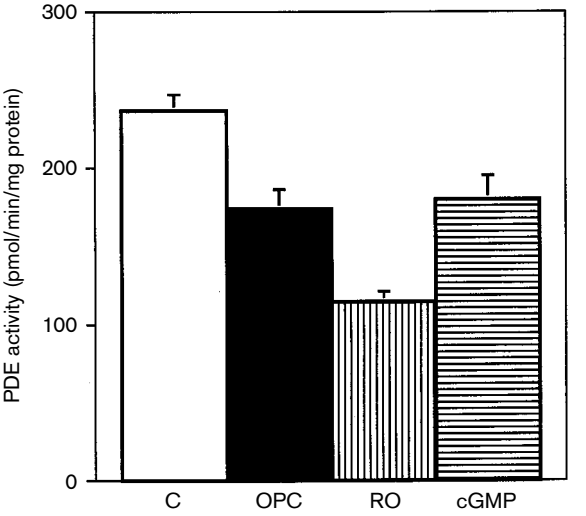

Figure 1. Effect of inhibitors on PDE activity in HSG cells. Homogenates were prepared from the tumor cells and assayed for PDE activity with or without inhibitors as described in Materials and methods. C (control), OPC (0.5 μM cilostamide), RO (10 μM rolipram) and cGMP (5 μM cGMP). Data are means ± SEM of three experiments.

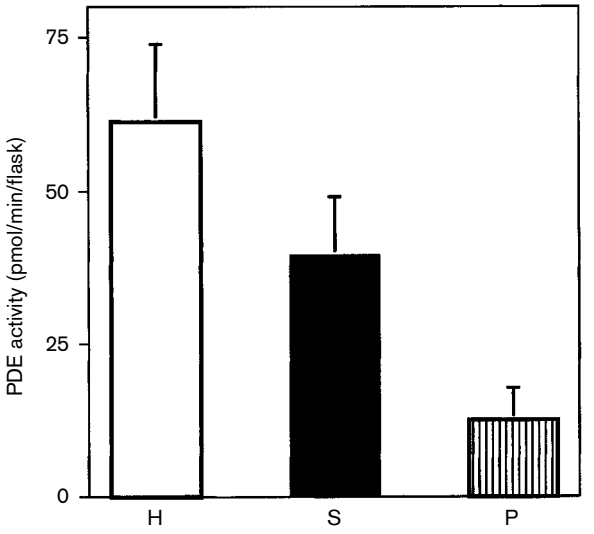

Figure 2. Distribution of PDE3 activity in HSG cells. Homogenates (H), particulate (P) and supernatant (S) fractions were prepared from HSG cells and assayed for PDE3 activity with or without 0.5 μM cilostamide as described in Materials and methods. Data are means ± SEM of three experiments.

In HSG cells, about 75% of the PDE3 activity was in the supernatant fractions and 25% was in the particulate fractions (Figure 2).

RT-PCR was performed on total RNA, using primer pairs specific for human PDE3A and 3B (Table 1). PDE3A and 3B mRNAs were detected in RNA from HSG cells (Figure 3); its nucleotide sequence was identical to that of PDE3A and 3B. No fragments were amplified in the absence of reverse transcriptase or RNA.

The PDE3-specific inhibitor, cilostamide, inhibited the growth of HSG cells (Figure 4).

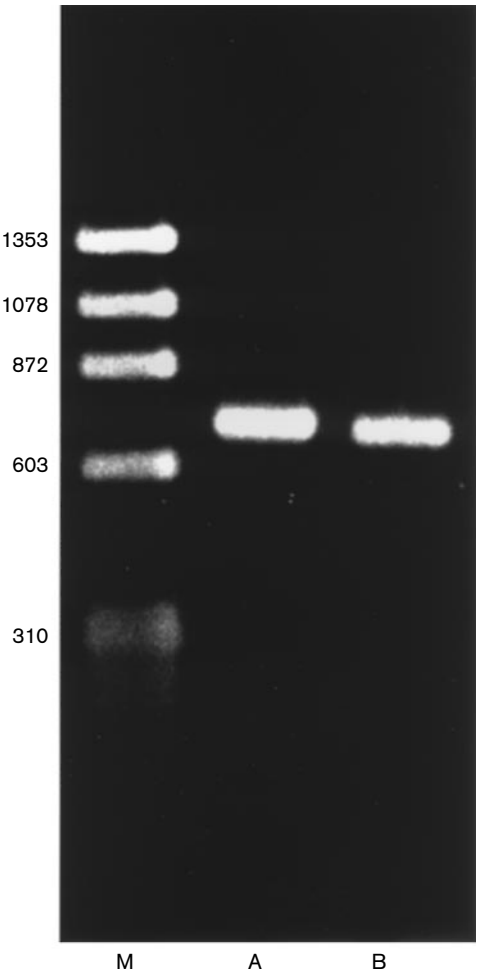

Figure 3. RT-PCR of PDE3 mRNAs in HSG cells. Total RNA was extracted as described in Materials and methods. cDNA was generated from 1 μg total RNA and amplified by PCR, using oligonucleotide primer sets based on sequences from PDE3A and 3B. The products were separated on agarose gels and photographed after ethidium bromide staining. PDE3A (A), PDE3B (B) and molecular markers (M).

Discussion

PDE3s are characterized by their sensitivity to several specific inhibitors, including cilostamide, enoximone and lixazinone, which are relatively selective for PDE3, with K_i and IC_{50} values at least 10- to 100-fold lower for PDE3 than for other PDE families.[1–3] When different PDEs were first identified, two types (now classified as PDE3 and PDE4) that exhibited a high affinity for cAMP were isolated from various tissues. cGMP is a competitive inhibitor of cAMP hydrolysis by PDE3, and inhibition of cAMP hydrolysis by cGMP also distinguishes PDE3 from PDE4 (cAMP-specific PDE) isoforms which exhibit a 'low K_m' for cAMP and are inhibited by rolipram but not by cGMP.[1–3] In extracts of HSG cells, inhibition of PDE activity with cilostamide, cGMP and rolipram indicated the presence of both PDE3 and PDE4 enzymes.

In platelets, PDE3 activity is predominantly cytosolic,[2,20] whereas in hepatocyte and adipocyte PDE3 activities is particulate,[2,21–23] and in heart, both particulate and cytosolic.[24] In two human hepatocellular carcinoma Hep3B and HuH7 cell lines, about 70% of the PDE3 activity was in the supernatant fractions and 30% was in the particulate fractions.[16] Our recent studies strongly suggested that determinants for association of PDE3 isoenzymes with intracellular membranes might be located in the N-terminal hydrophobic region, perhaps within the

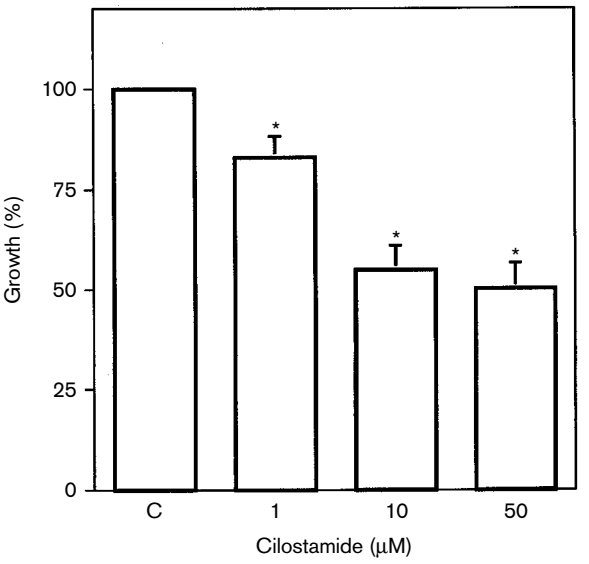

Figure 4. Effect of cilostamide on the growth of HSG cells. Cells were plated in 24-well plates and cultured with the indicated concentrations of cilostamide. The cells were counted as described in Materials and methods. Data are means ± SEM of three experiments. Significantly different from the control ($^{*}p<0.01$).

predicted transmembrane segments.[25,26] Results from the distribution of PDE3 activities in HSG cells suggest that there are two types of PDEs in these cells, with and without membrane-association domains.

PDE3A mRNAs are relatively highly expressed in rat heart, rat aorta, human heart and human aorta; PDE3B mRNAs, in rat and 3T3-L1 adipocytes and rat adipose tissue.[1–3,27] We previously reported that PDE3A and 3B were expressed in human hepatocellular carcinoma Hep3B and HuH7 cells, and suggested that both PDE3s play important roles in these cells.[16] Using specific oligonucleotide primers, both PDE3A and 3B mRNAs were detected by RT-PCR in RNA from HSG cells. These results were consistent with the inhibition of HSG PDE activity by cilostamide and cGMP.

To establish the role of PDE3 in HSG cells, we studied the effect of a PDE3-specific inhibitor, cilostamide, on the growth of HSG cells. Cilostamide inhibited the growth of HSG cells. Specific inhibitors of PDE3 inhibit proliferation of several types of cells.[13,28,29] Thus, inhibition of PDE3, which presumably results in an increase in cAMP (and/or cGMP), blocks proliferation of several types of cells, including HSG malignant submandibular cells. In many tumor cells, which have much lower basal levels of cAMP than normal cells, cAMP is a negative messenger for proliferation.[30] Although various cAMP elevating agents have previously been found to inhibit tumor cell growth *in vitro*,[30–33] the mechanisms involved in cAMP-dependent inhibition of cell proliferation are not completely understood.

Our results indicate that PDE3s may be important in the growth of HSG cells and may be potential new targets for antiproliferative therapies.

Conclusion

PDE 3s have been characterized in human neoplastic submandibular gland intercalated duct HSG cells. PDE3A and 3B mRNAs were detected by RT-PCR in RNA from HSG cells. The PDE3-specific inhibitor, cilostamide, inhibited the growth of HSG cells. Our results indicate that PDE3s may be important in the growth of HSG cells and PDE3 inhibitors would be useful therapeutic drugs for malignant tumors in the submandibular gland.

References

1. Manganiello VC, Murata T, Taira M, Belfrage P, Degerman, E. Perspectives in biochemistry and biophysics. Diversity in cyclic nucleotide phosphodiesterase isoenzyme families. *Arch Biochem Biophys* 1995; **322**: 1–13.
2. Manganiello VC, Taira M, Degerman E, Belfrage P. Type III cGMP-inhibited cyclic nucleotide phosphodiesterases (PDE3 gene family). *Cell Signal* 1995; **7**: 445–55.
3. Degerman E, Belfrage P, Manganiello VC. Structure, localization, and regulation of cGMP-inhibited phosphodiesterase (PDE3). *J Biol Chem* 1997; **272**: 6823–6.
4. Meacci E, Taira M, Moos Jr M, *et al*. Molecular cloning and expression of human myocardial cGMP-inhibited cAMP phosphodiesterase. *Proc Natl Acad Sci USA* 1992; **89**: 3721–25.
5. Taira M, Hockman SC, Calvo JC, Taira M, Belfrage P, Manganiello VC. Molecular cloning of the rat adipocyte hormone-sensitive cyclic GMP-inhibited cyclic nucleotide phosphodiesterase. *J Biol Chem* 1993; **268**: 18573–9.
6. Fisher DA, Smith JF, Pillar JS, St Denis SH, Cheng JB. Isolation and characterization of PDE8A, a novel human cAMP-specific phosphodiesterase. *Biochem Biophys Res Commun* 1998; **246**: 570–7.
7. Hayashi M, Matsushima K, Ohashi H, *et al*. Molecular cloning and characterization of human PDE8B, a novel thyroid-specific isozyme of 3′,5′-cyclic nucleotide phosphodiesterase. *Biochem Biophys Res Commun* 1998; **250**: 751–6.
8. Soderling SH, Bayuga SJ, Beavo JA. Cloning and characterization of a cAMP-specific cyclic nucleotide phosphodiesterase. *Proc Natl Acad Sci USA* 1998; **95**: 8991–6.
9. Fisher DA, Smith JF, Pillar St JS, Denis SH, Cheng JB. Isolation and characterization of PDE9A, a novel human cGMP-specific phosphodiesterase. *J Biol Chem* 1998; **273**: 15559–64.
10. Fujishige K, Kotera J, Michibata H, *et al*. Cloning and characterization of a novel human phosphodiesterase that hydrolyzes both cAMP and cGMP (PDE10A). *J Biol Chem* 1999; **274**: 18438–45.

11. Fawcett L, Baxendale R, Stacey P, *et al*. Molecular cloning and characterization of a distinct human phosphodiesterase gene family: PDE11A. *Proc Natl Acad Sci USA* 2000; **97**: 3702–7.
12. Miki T, Taira M, Hockman S, *et al*. Characterization of the cDNA and gene encoding human PDE3B, the cGIP1 isoform of the human cyclic GMP-inhibited cyclic nucleotide phosphodiesterase family. *Genomics* 1996; **36**: 476–85.
13. Ekholm D, Hemmer B, Gao G, Vergelli M, Martin R, Manganiello V. Differential expression of cyclic nucleotide phosphodiesterase 3 and 4 activities in human T cell clones specific for myelin basic protein. *J Immunol* 1997; **159**: 1520–9.
14. Seybold J, Newton R, Wright L, *et al*. Induction of phosphodiesterses 3B, 4A4, 4D1, 4D2, and 4D3 in Jurkat T-cells and in human peripheral blood T-lymphocytes by 8-bromo-cAMP and Gs-coupled receptor agonists. *J Biol Chem* 1998; **273**: 20575–88.
15. Cheung PP, Xu H, McLaughlin MM, Ghazaleh FA, Livi GP, Colman RW. Human platelet cGI-PDE: expression in yeast and localization of the catalytic domain by deletion mutagenesis. *Blood* 1996; **88**: 1321–9.
16. Murata T, Taira M, Manganiello VC. Differential expression of cGMP-inhibited cyclic nucleotide phosphodiesterases in human hepatoma cell lines. *FEBS Lett* 1996; **390**: 29–33.
17. McGurk M, Williams RG, Calman FMB. A clinical approach to malignant tumours. In: Norman JED, McGurk M, eds. *Color atlas and text of the salivary glands. Diseases, disorders and surgery*. London: Mosby-Wolfe 1995: 181–96.
18. Shirasuna K, Sato M, Miyazaki T. A neoplastic epithelial duct cell line established from an irradiated human salivary gland. *Cancer* 1981; **48**: 745–52.
19. Degerman E, Moos Jr M, Rascón A, *et al*. Single-step affinity purification, partial structure and properties of human platelet cGMP inhibited cAMP phosphodiesterase. *Biochim Biophys Acta* 1994; **1205**: 189–98.
20. Grant PG, Colman RW. Purification and characterization of a human platelet cyclic nucleotide phosphodiesterase. *Biochemistry* 1984; **23**: 1801–7.
21. Degerman E, Belfrage P, Newman AH, Rice KC, Manganiello VC. Purification of the putative hormone-sensitive cyclic AMP phosphodiesterase from rat adipose tissue using a derivative of cilostamide as a novel affinity ligand. *J Biol Chem* 1987; **262**: 5797–807.
22. Pyne NJ, Cooper ME, Houslay MD. The insulin- and glucagon-stimulated 'dense-vesicle' high-affinity cyclic AMP phosphodiesterase from rat liver. Purification, characterization and inhibitor sensitivity. *Biochem J* 1987; **242**: 33–42.
23. Boyes S, Loten EG. Purification of an insulin-sensitive cyclic AMP phosphodiesterase from rat liver. *FEBS Lett* 1988; **174**: 303–9.
24. Harrison SA, Reifsnyder DH, Gallis B, Cadd GG, Beavo JA. Isolation and characterization of bovine cardiac muscle cGMP-inhibited phosphodiesterase: a receptor for new cardiotonic drugs. *Mol Pharmacol* 1986; **29**: 506–14.
25. Leroy MJ, Degerman E, Taira M, *et al*. Characterization of two recombinant PDE3 (cGMP-inhibited cyclic nucleotide phosphodiesterase) isoforms, RcGIP1 and HcGIP2, expressed in NIH 3006 murine fibroblasts and Sf9 insect cells. *Biochemistry* 1996; **35**: 10194–202.
26. Kenan Y, Murata T, Shakur Y, Degerman E, Manganiello VC. Functions of the N-terminal region of cyclic nucleotide phosphodiesterase 3 (PDE3) isoforms. *J Biol Chem* 2000; in press.
27. Reinhardt RR, Chin E, Zhou J, *et al*. Distinctive anatomical patterns of gene expression for cGMP-inhibited cyclic nucleotide phosphodiesterases. *J Clin Invest* 1995; **95**: 1528–38.
28. Marcoz P, Prigent AF, Lagarde M, Némoz G. Modulaton of rat thymocyte proliferative response through the inhibition of different cyclic nucleotide phosphodiesterase isoforms by means of selective inhibitors and cGMP-elevating agents. *Mol Pharmacol* 1993; **44**: 1027–35.
29. Pan X, Arauz E, Krzanowski JJ, Fitzpatrick DF, Polson, JB. Synergistic interactions between selective pharmacological inhibitors of phosphodiesterase isozyme families PDE III and PDE IV to attenuate proliferation of rat vascular smooth muscle cells. *Biochem Pharmacol* 1994; **48**: 827–35.
30. Drees M, Zimmermann R, Eisenbrand G. 3′,5′-Cyclic nucleotide phosphodiesterase in tumor cells as potential target for tumor growth inhibition. *Cancer Res* 1993; **53**: 3058–61.
31. Bertram JS, Faletto MB. Requirements for and kinetics of growth arrest of neoplastic cells by confluent 10T1/2 fibroblasts induced by a specific inhibitor of cyclic adenosine 3′:5′-phosphodiesterase. *Cancer Res* 1985; **45**: 1946–52.
32. Lichtner RB, Goka TJ, Butcher RW, Nicolson GL. Direct effects of the pyrimido-pyrimidine derivative RA 233 (Rapenton) on rat 13762NF mammary tumor cell clones *in vitro*. *Cancer Res* 1987; **47**: 1870–7.
33. Lando M, Abemayor E, Verity MA, Sidell N. Modulation of intracellular cyclic adenosine monophosphate levels and the differentiation response of human neuroblastoma cells. *Cancer Res* 1990; **50**: 722–7.

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