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Anti-leukemic activity of DNA methyltransferase inhibitor procaine targeted on human leukaemia cells

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Abstract: Chromatin remodeling in DNA is fundamental to gene expression, DNA replication and repair processes. Methylation of promoter regions of tumor-suppressor genes and histone deacetylation leads to gene silencing and transcriptionally repressive chromatin. For the past few decades DNA methylation agents became very attractive as the targets for cancer therapy. The purpose of this work was to examine the effects of DNMT inhibitor procaine on growth inhibition, apoptosis and differentiation of human leukaemia cells. The changes in expression of genes, proteins and histone modifications caused by procaine were evaluated under different treatments. We demonstrated that procaine arrests growth of human leukaemia cells and in combination with all-trans retinoic acid (ATRA) induces cancer cell differentiation. Procaine causes reduction of expression of DNA methyltransferases as well. The treatment of human leukaemia cells with procaine increase the expression of molecules associated with differentiation (CD11b, E-cadherin, G-CSF) and apoptosis (PPARγ). Moreover, the examined DNMT inhibitor enhances certain gene transcription activation via chromatin remodelling – the changes in histone H3K4(Me)3 and H3K9Ac/S10P modifications were detected. Our results suggest, that DNMT inhibitor – procaine, can be used for further investigations on epigenetic differentiation therapy of leukaemia cells especially when used in combination with retinoic acid.

Keywords: DNMTI, procaine, chromatin, remodeling

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1 Introduction

Epigenetic changes such as DNA methylation and histone deacetylation play an important role in gene expression regulation. Methylation of promoter regions of tumour-suppressor genes and histone deacetylation leads to gene silencing and transcriptionally repressive chromatin [1-3].

DNMT inhibitors, specifically cytidine analogues, are potent anticancer reagents in cell culture models in a variety of human cancers. The main disadvantage of cytidine analogues is their incorporation into the entire genome and their lack of gene specificity, and also their toxicity. These effects could be avoided by developing DNMT inhibitors that could inhibit DNMT catalytic site directly [4].

A few non-nucleoside inhibitors of DNA methyltransferases have been reported, such as CP-4200, SGI-110 (analogues of azacytidine and decitabine, respectively), the oligonucleotides MG98 and miR29a, RG108, epigallocatechin gallate (EGCG) [4-7].

During recent years procaine, an anaesthetic, and the anti-arrhythmia drug procainamide have been under investigation in this regard. It was found that procaine causes global DNA hypomethylation, demethylation and re-expression of a CpG-island-associated gene *retinoic acid receptor beta 2 (RARβ2)* and *glutathione S-transferase pi gene* (*GSTP1)*, and growth inhibition in breast cancer cells [3, 7-8]. It was showed by Tada and co-workers (2007) that PCA treatment led to the partial demethylation and restoration of mRNA expression of genes (*p16INK4a*, *HAI-2/PB*, *14-3-3-sigma*, and *NQO1)* in hepatoma cells that increased growth-inhibitory effects [8-11].

Gao and co-workers (2009) demonstrated promoter demethylation of Wnt inhibitory factor 1 (WIF-1), restoration of WIF-1 expression, and under expression of cytosolic beta-catenin protein and T Cell Factor (TCF) reporter activity, after procaine and procainamide treatment in lung cancer cell lines. These results provide the first evidence that procaine and procainamide reactivate WIF-1 in cancer cells and downregulate the Wnt canonical pathway [9].

During our study we examined the growth inhibitory effects of procaine in NB4 leukaemia cells. It was noted that, procaine arrests growth of human leukaemia cells and in combination with ATRA induces differentiation. Procaine causes reduction of expression of DNA methyltransferases. Human leukaemia cells under the treatment with procaine increase the expression of molecules associated with differentiation (E-cadherin) and apoptosis (PPARγ). Moreover, this DNMT inhibitor enhances transcription activating histone modifications, i.e. H3K4(Me)$_3$ and H3K9Ac/S10P.

We suggest that procaine can be used for further detailed investigations on epigenetic and differentiation therapy especially when used in combination with retinoic acid or similar agent. It can be promising agent for anticancer therapy.

2 Materials and methods

2.1 Materials

Procaine and ATRA were purchased from Sigma Chemical Co. (St. Louis, MO). The stock solutions of procaine 100 mM in DMSO and ATRA 2 mM in ethanol were stored at -20°C. Rabbit polyclonal antibodies against pan-cadherin (ab16505), mouse monoclonal antibodies against GAPDH (ab8245), and goat monoclonal antibodies against H3K9Ac/S10P (ab8898) were obtained from Abcam, Cambridge, UK. Antibodies against histone H3K4Me3 were obtained from Upstate, Lake Placid, USA. Goat polyclonal antibodies against DNMT1 (C-17) were obtained from Santa Cruz Biotechnology, Inc. Mouse monoclonal anti-human CD11b, C3bi receptor conjugated with PE and goat anti-rabbit (or anti-mouse) HPR-linked secondary antibodies were from Dako Cytomation A/S, Lustrum, Denmark.

2.2 Cell culture

The human promyelocytic leukaemia NB4 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Grand Island, NY) at 37°C in a humidified 5% CO_2 atmosphere. In each experiment, logarithmically growing cells were seeded in 5 ml of medium at a density 5×10^5 cells/ml. In experiments, cells were exposed to DNMT inhibitor alone or in combination with differentiation inducer 1 μM ATRA for 24, 48 or 72 h. Treated cells were cultured and harvested at time points indicated.

2.3 Cell proliferation, differentiation and apoptosis assays

Cell proliferation was evaluated by the trypan blue exclusion test. Viable (not coloured) and dead (blue coloured) cell number was determined by counting in a haemocytometer. The growth inhibition was calculated from: [(Cx-Co) – (Tx-To)]/ (Cx-Co) × 100, where Co, Cx, To and Tx represents the total number of cells/ml in untreated (C) and treated (T) cultures at days 0 and x (2 or 3), respectively. At least 400 cells were scored for each determination.

For differentiation analysis, control (untreated) and treated NB4 cells (5×10^5 cells/sample) were washed twice in PBS (pH 7.4), then exposed to mouse monoclonal anti-human CD11b conjugated with PE for 30 min in the dark at 4°C. Cells were washed with PBS, fixed in PBS containing 2% paraformaldehyde for 30 min on ice and pellet was suspended in PBS. Eight thousands events were analysed for each sample by immunofluorescence in FACSaria (BD Bioscences with BD FACS Diva software). Proliferating cells were used as a control. Negative control was performed using an isotype-specific mouse PE-conjugated IgG1 (Santa Cruz Biotechnology, Inc., Heidelberg, Germany).

Apoptosis were monitored by quantification of cellular DNA content after staining with PI. Control and treated cells were collected by centrifugation, suspended in PBS and fixed in ice-cold 70% ethanol (ratio 1:10) for 24 h at -20°C. After centrifugation at 500 *g* for 5 min, cells were suspended in PBS containing PI (50 μg/ml) and RNAse (0.2 mg/ml) and incubated at room temperature for 30 min. The tubes were then kept at 4°C in dark until flow cytometric analysis in FACSCalibur (Becton-Dickinson, USA). Apoptotic cells were quantified on PI histogram as a hypodiploid peak (SubG1) and the data was registered on a logarithmic scale.

For statistical analysis, Student's t test was used. Results are mean ± SEM ($n = 3$).* $p < 0.05$, ** $p < 0.001$ and *** $p < 0.0001$.

2.4 Protein isolation, gel electrophoresis and western blot analysis

Briefly, cells were harvested and washed twice in PBS (pH 7.5), suspended to 3×10^7 cells/ml in lysis solution (150 mM NaCl, 10 mM Tris/HCl (pH 7.5), 5 mM EDTA, 1% Nonidet P-40, 1 mM PMSF, 5 mM NaF, 1 mM Na_3VO_4, and 1x protease inhibitor cocktail (Roche)). The lysates were homogenized and incubated for 15 min at 0°C and then centrifuged at 15,000 *g* for 30 min. Supernatants were collected as soluble

protein fraction. For SDS-PAGE, to the protein samples equal volumes of 2x SDS loading buffer (Thermo Fisher Scientific Inc., Waltham, MA, USA) was added and boiled for 5 min. The supernatants were immediately subjected to electrophoresis or frozen at -76°C. For electrophoresis, we used an 8–16% polyacrylamide gradient gel in Tris-glycine electrophoresis buffer. After SDS/PAGE, proteins were transferred to a PVDF membrane (Immobilon P, Millipore, Bedford, MA) and then the filters were blocked with 5% BSA dissolved in PBS containing 0.18% Tween-20 by incubation overnight at 4°C. After washing in PBS-Tween-20, the filters were probed with primer antibodies against pan-cadherin (1:500 dilution), DNMT1 (1:1,000 dilution), H3K9Ac/S10P (1:8,000 dilution), $H3K4Me_3$ (1:7,000 dilution) or GAPDH (at 1:10,000 dilution) for 1 h at room temperature. Primary antibodies were prepared in solution containing 0.18% Tween-20, 0.35 M NaCl and 1% BSA. The filters were subsequently washed four times with PBS-Tween-20 and then incubated with HRP-linked goat anti-mouse or goat anti-rabbit secondary antibody for 1 h at room temperature. Immunoreactive bands were detected by enhanced chemiluminescence using ECL™ Western blotting detection reagents (GE Healthcare Life Sciences, USA) according to the instructions of the manufacturer.

2.5 RTPCR analysis

Total RNA was obtained using the Trizol (Invitrogen, Carlsbad, USA) method and cDNA was prepared using the RevertAid ™ Premium First strand cDNA synthesis kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) following the manufacturer's protocols. Quantitative RT-PCR was performed with Maxima® SYBR Green qPCR Master Mix (Thermo Scientific, EU) on the Rotor-Gene 6000 system (Corbett Life Science, Sydney, Australia). The amount of mRNA was normalized to HPRT1. The relative gene expression was calculated by a comparative threshold cycle delta-delta Ct method. Results normalised to GAPDH.

Results presented as% = **100 × (RQ (Control) /RQ (xtreatment)).**

The primers (5'-3' orientation) used in RTPCR reactions listed in Table 1.

Cycling conditions: 95°C 5 min, 95°C 30 s, annealing 30 s, 72°C 30 s, 72°C 10 min, +4°C. The products were electrophoresed on 3% agarose gel, stained with ethidium bromide, and photographed.

3 Results

3.1 Cell growth, apoptosis and differentiation evaluation in promyelocytic leukaemia NB4 cells treated with procaine and differentiation inducer ATRA

In order to estimate the anti-proliferative effects, NB4 cells were treated with 3 or 5 μM Procaine, differentiating agent 1 μM ATRA alone and with combination of 1 μM ATRA and 3 μM Procaine during 3 days. DNMTI decitabine (0.5 μM) was used as a control demethylating agent for cell treatment.

Table 1. The primers used in this study.

Gene	Primers	Product size (bp)	Annealing temperature [°C]
PPARg	F-GCTCTAGAATGACCATGGTTGAC	269	64
	R–TAAGGTGGAGATGCAGGCT		
Cadherin	F-TCCTGGGCAGAGTGAATTTT	94	55
	R-ACACCATCTGTGCCCACTTT		
HPRT1	F-TGGCGTGTGATTAGTGATG	88	60
	R-ACCCTTTCCCAAATCCTCAGC		
DNMT1	F-AGGGAAAAGGGAAGGGCAAG	120	60
	R-TCTTTTGTGTAGGTCCCAGGC		
DNMT3A	F-CAGCGTCACACAGAAGCATATCC	433	60
	R-GGTCCTCACTTTGCTGAACTTGG		
DNMT3B	F-CCTGCTGAATTACTCACGCCCC	421	60
	R-GTCTGTGTAGTGCACAGGAAA		
G-CSF	F-GCTGGGCAAGGTGGCGTAGAACGC	442	64
	R-CTGGGACAGTGCAGGAAGCCACC		

The anti-proliferative effect of procaine was evaluated after staining with trypan blue: only dead cells were coloured in blue. Procaine inhibited growth of NB4 cells in a dose and time-dependent manner (Fig.1A). By contrast, ATRA had only minor anti-proliferative and growth inhibitory (29%) effects at day 3. Combined treatment with ATRA and procaine inhibited cell growth 100% at day 2-3.

Apoptotic effect of procaine on NB4 cells were monitored by quantification of cellular DNA content after staining with Propidium Iodide by flow cytometry. Procaine at a dose of 3 μM induced apoptotic cell death (to 17%) and at a dose of 5 μM induced apoptotic cell death (to 30%) as was determined by flow cytometry after cell staining with PI on day 3 (Fig. 1B), whereas ATRA alone or in combination with procaine induced apoptosis only up to 13-14% as compared to the spontaneous apoptosis in non-treated control (5%). Decitabine at a dose of 0.5 μM induced apoptosis to 17%, like 3 μM procaine dose did (Fig. 1B).

Using the same experimental conditions, we analyzed the differentiation effects of procaine in NB4 cells on

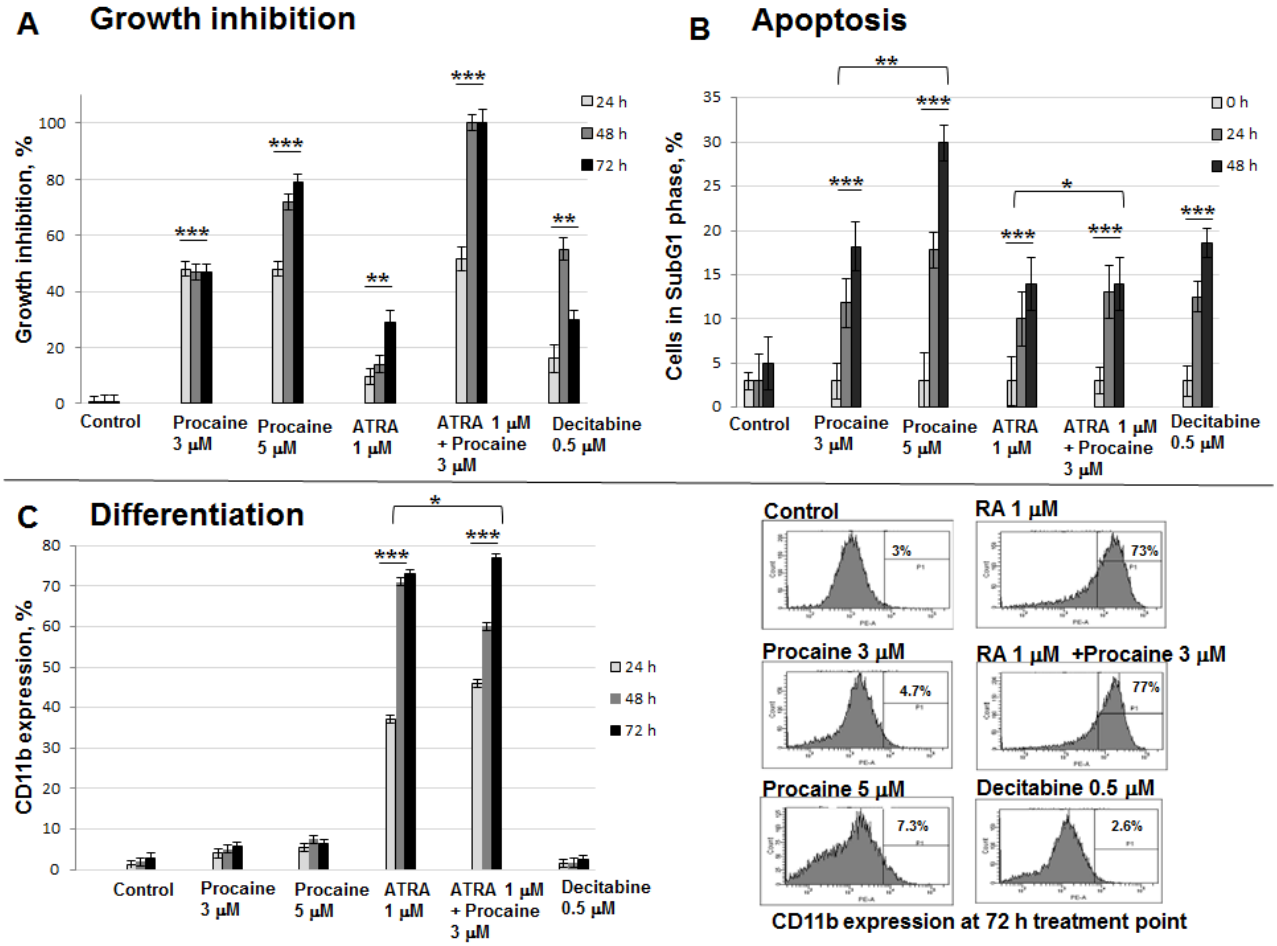

Fig.1. Effects of procaine on Growth inhibition, differentiation and apoptosis of NB4 cells. Cells were exposed to 3 or 5 μM of procaine alone, 1 μM ATRA alone, combination of 1 μM ATRA and 3 μM procaine, 0.5 μM decitabine alone for 3 days.

A- **Dose-dependent effects of procaine on the growth of leukemia cell lines NB4.** Control and treated cells were subjected to counting following staining with 0.2% tripan blue. Viable (uncolored) and dead (blue colored) cell number was determined by counting in a haemocytometer. The growth inhibition was calculated from: [(Cx-Co) – (Tx-To)] / (Cx-Co) × 100%, where Co, Cx, To and Tx represent the total number of cells/ml in untreated (C) and treated (T) cultures at days 0 and x (2 or 3), respectively. At least 400 cells were scored for each determination. Results are mean ± SEM (n = 3).* p < 0.05, ** p < 0.001 and *** p < 0.0001.

B- **Dose-dependent effects of procaine on the apoptosis of leukemia cell lines NB4.** Control cells and treated cells for 48 h were analyzed for the apoptosis. Apoptosis was determined as a hypodiploid peak (subG1) from flow cytometric analysis of PI stained cells. Apoptosis is presented as a percentage of the total events collected. Results are mean ± SEM (n = 3).* p < 0.05, ** p < 0.001 and *** p < 0.0001.

C- **nfluence of procaine on expression of CD11b in NB4 cells.** Control cells and treated cells for 72 h were analyzed for the early myeloid cell surface differentiation marker CD11b by flow cytometry. Results are mean ± SEM (n = 3).* p < 0.05, ** p < 0.001 and *** p < 0.0001Representative FACS-generated histograms of CD11b of control and treated cells for 72 h are given as well.

the expression of an early marker of myelomonocytic maturation, CD11b during 72 h, i.e., before the appearance of morphological cell maturation. Flow cytometry analysis showed that procaine and decitabine alone did not induce the expression of an early differentiation marker CD11b (Fig. 1, C, D), but combined treatment ATRA with procaine increased CD11b expression up to 78% at day 3.

3.2 Procaine alone and in combination with ATRA effectively inhibit DNMT1, 3A and 3B expression

In order to estimate the demethylating potential of procaine and its combination with ATRA in NB4 leukaemia cells, we verified the ability to change expression of the chromatin-modifying enzyme DNMT1, which is mostly overexpressed in leukaemia in association with promoter hypermethylation of a number of genes involved in cell proliferation, apoptosis or differentiation processes. To determine effects of procaine or its combination with ATRA on DNMT expression level in NB4 cells, we performed gene (DNMT1, DNMT3A and DNMT3B) expression experiments (RTPCR) and Western blotting of DNMT1 protein (Fig. 2)

Procaine alone at doses of 3 and 5 μM inhibited gene expression up to 10-40% after 24 h of treatment (Fig. 2A). At 96 h of treatment this effect was lower in comparison with 24 h of procaine. The DNMT1 protein expression level was lowest after 96 h of treatment with 3 and 5 μM of procaine (Fig. 2B).

The combined treatment of ATRA and procaine showed DNMT1 gene and protein expression down-regulation up to 50% at 24, 96 h points of treatment, the same as ATRA and Decitabine treatments (Fig. 2A, B).

DNMT3A gene expression was down-regulated after treatment with procaine or ATRA alone, but no significant inhibition was determined after combined procaine and ATRA treatment or Decitabine (Fig. 2A).

DNMT3B gene expression was inhibited in all cases of treatments, with greater manner after ATRA and its combination with procaine (up to 40%) (Fig. 2A).

3.3 Procaine alone results in PPARγ gene expression activation and only in combined treatment with ATRA enhance G-CSF gene expression

The *G-CSF gene* encodes a hematopoietic colony-stimulating factor (CSF) that promotes growth, differentiation, and survival of neutrophilic granulocytes. To determine procaine effects on G-CSF expression we performed RTPCR analysis. Procaine alone had no effect on *G-CSF* gene expression (Fig. 3A). Combined treatment with ATRA and procaine up-regulated expression of *G-CSF* at 24-96 h points, as well as after ATRA treatment alone (Fig. 3A). This correlated with differentiation marker CD11b expression in cells treated with ATRA alone or in combination with procaine.

Peroxisome proliferator-activated receptor gamma (PPARγ) has been demonstrated to play an important role in the regulation of cell apoptosis and differentiation. To show PPARγ gene expression changes after the treatments, we performed PCR analysis. PPARγ gene expression was up-regulated up to 48% after 3 μM procaine treatment and up to 20% after 5 μM procaine treatment at 96 h point

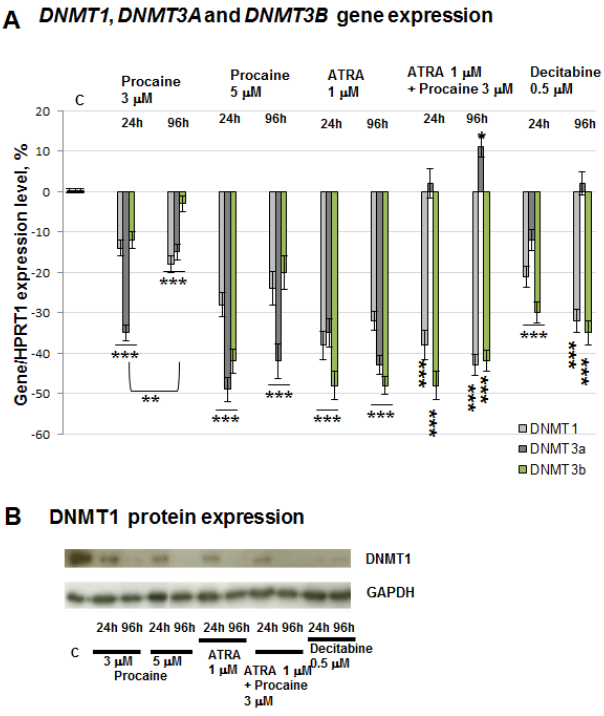

Fig.2. Expression of DNMTs in response to procaine treatment. Cells were exposed to 3 or 5 μM of procaine alone, 1 μM ATRA alone, combination of 1 μM ATRA and 3 μM procaine, 0.5 μM decitabine alone for 4 days.

A- Expression levels of DNMT1, DNMT3A **and** DNMT3B were determined by RTPCR analysis. The amount of mRNA was normalized to HPRT1. The relative gene expression was calculated by a comparative threshold cycle delta-delta Ct method and results are given as% changes to control cells. Results are mean ± SEM (n = 3). * p < 0.05, ** p < 0.001 and *** p < 0.0001.

B- DNMT1 protein expression. Equal amounts of proteins from cell lysates were electrophoresed, and Western blot analysis was performed using antibodies against DNMT1 and GAPDH (as a loading control). Intensity of protein bands was measured using ImageJ program. Results are given as% changes to control cells. Results are mean ± SEM (n = 3).* p < 0.05, ** p < 0.001 and *** p < 0.0001.

(Fig. 3B). The changes in PPARγ gene expression were not significant (5-10%) after cell treatments with agents- ATRA or decitabine alone or in combined (ATRA and procaine) treatments (Fig. 3B).

3.4 Reactivation of E-cadherin expression in NB4 cells in response to treatment with DNMTI and ATRA

Cells exposed to procaine (3 and 5 μM) for 24-96 h had increased expression of E-Cadherin up to 40-60%. Fig. 4 presents *Cadherin* gene and protein expression levels in NB4.

ATRA alone or in combination with procaine caused very small increase in *cadherin* gene expression. Decitabine (0.5 μM) induced increase in Cadherin protein expression level up to 40% and procaine even up to 70% at 24 h treatment (Fig. 4, B).

3.5 Procaine induced epigenetic changes mediated by histone H3 modifications

To test epigenetic changes in chromatin structure we tested two histone modifications: trimethylation of H3 K4 and acetylation/phosphorylation of H3 K9/S10. Both modifications represent active chromatin structure. NB4

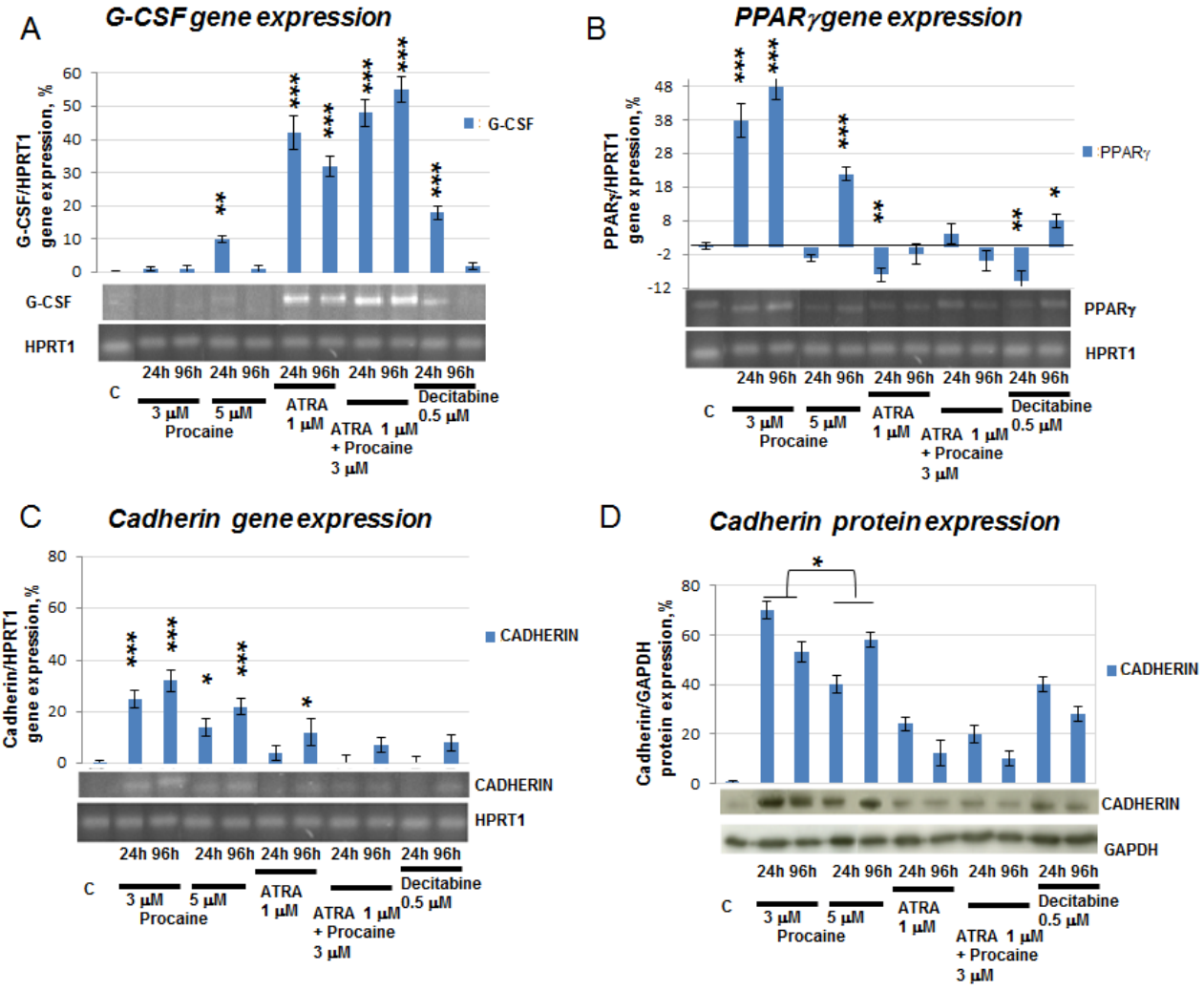

Fig.3. Effects of procaine on G-CSF, PPARγ **and E-cadherin expression.** Cells were exposed to 3 or 5 μM of procaine alone, 1 μM ATRA alone, combination of 1 μM ATRA and 3 μM procaine, 0.5 μM decitabine alone for 4 days.
G-CSF (**A**), *PPARγ* (**B**) and *Cadherin* (**C**) gene expression were determined by RTPCR analysis. The amount of mRNA was normalized to HPRT1. The relative gene expression was calculated by a comparative threshold cycle delta-delta Ct method and results are given as% changes to control cells. Results are mean ± SEM ($n = 3$).* $p < 0.05$, ** $p < 0.001$ and *** $p < 0.0001$.
Cadherin protein expression (**D**) was determined by Western blot analysis. Equal amounts of proteins from cell lysates were electrophoresed, and Western blot analysis was performed using antibodies against cadherin and GAPDH (as a loading control). Intensity of protein bands was measured using ImageJ program. Results are given as% changes to control cells. Results are given as mean ±S.E.M. ($n = 3$).

cells were treated with ATRA and procaine alone or in combination (Fig. 4). The results showed that procaine alone (3 μM) caused H3 K4 Methylation up to 18% after 96 h of treatment. The higher concentration of procaine (5 μM) showed increase in H3 K4 Methylation level up to 17% at 24 h point. ATRA alone or in combination with procaine had less effects on H3 K4 Methylation compared with procaine alone.

Procaine or ATRA alone in NB4 cells caused increased expression of H3 K9 Acetylation/S10 phosphorylation up to 50-60% (Fig. 5). Combination of ATRA and procaine showed H3 K9 Acetylation/S10 phosphorylation up to 40% at 96 h of treatment in comparison with control NB4 cells.

4 Discussion

Methylation-associated silencing of tumour suppressor genes is recognized as being a molecular hallmark of human cancer [4]. Procaine and procainamide, a derivative of procaine, are demonstrated to interact with CpG rich DNA regions and lead to DNA demethylation of genes such as the *RAR beta 2* gene, restoring gene expression of epigenetically silenced genes. Procaine can be very attractive for cancer treatment since it binds to DNA but is not incorporated into it. So, procaine treatments could have less side effects than Decitabine and others, which are currently used for the treatment of AML and MDS. Procaine's long-established and safe use as a local anaesthetic, with well-known characteristics, may stimulate its transition to preclinical and early clinical trials for epigenetics-based cancer treatments [8-11].

In recent years, many different DNMT inhibitors have been developed, and multiple molecular mechanisms through which these agents exert anti-cancer effects have been identified. It was demonstrated by a large number of clinical trials that DNMT inhibitors seem to be promising for treating several types of cancer. Indeed, combining of epigenetic therapy with conventional chemotherapy would be one of the major challenges in the future for developing effective strategies of cancer therapy [2-4].

DNA methyltransferase -enzyme family composed of DNMT1, DNMT3a and DNMT3b- adds a methyl (CH_3) group on cytosines of CG dinucleotides and affects gene expression. DNMT1 is known as maintenance DNMT which is responsible for the exact copying of the DNA methylation pattern on the neo-synthesized strand during DNA replication. DNMT1 expression is tightly regulated during the cell cycle by several mechanisms and maximal expression occurs during S phase [12-13]. The *de novo* DNA methyltransferases DNMT3a and DNMT3b are responsible

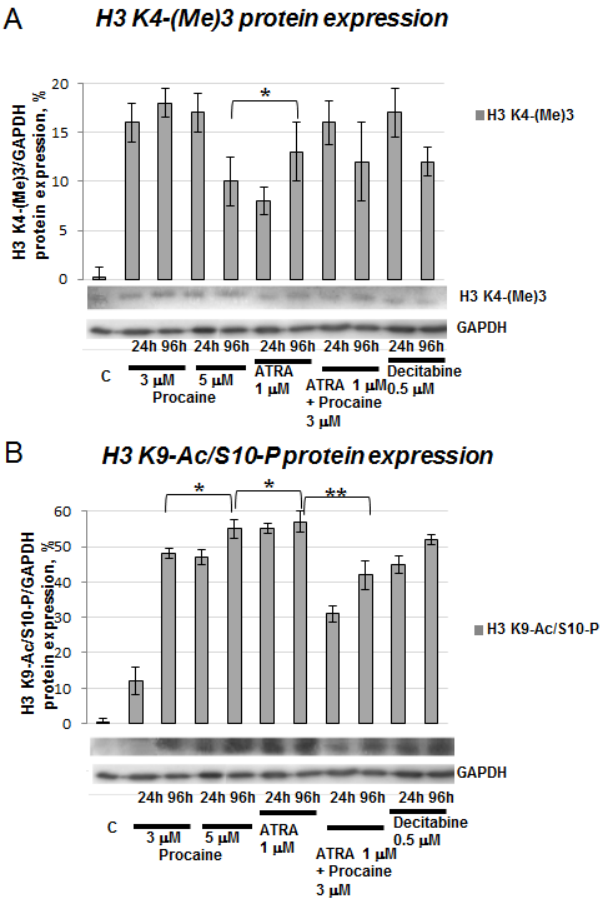

Fig.4. Procaine modified H3K4 Methylation (A) and H3 K9 Acetylation/S10 phosphorylation (B) status in NB4 cells. Cells were exposed to 3 or 5 μM of procaine alone, 1 μM ATRA alone, combination of 1 μM ATRA and 3 μM procaine, 0.5 μM decitabine alone for 4 days. Equal amounts of proteins from cell lysates were electrophoresed, and Western blot analysis was performed using antibodies against H3K9Ac/S10P, H3K4Me_3 and GAPDH (as a loading control). Intensity of protein bands was measured using ImageJ program. Results are given as% changes to control cells. Results are given as mean ±S.E.M. (n = 3). * p < 0.05, ** p < 0.001 and *** p < 0.0001.

for the establishment of DNA methylation patterns during development [12-13]. In this study we demonstrated that DNMTI procaine inhibited DNMT1 gene expression after 24 h of treatment. This effect was enhanced in combined treatment of procaine and ATRA. The same effect was observed in the DNMT3b gene expression. DNMT3a gene expression on the other hand was down-regulated by procaine alone whilst the combined treatment with ATRA had no effect. We examined the growth effects of procaine on NB4 cells, in addition to its DNA-demethylation properties. This is crucial in future use of this drug in any cancer therapy based on epigenetics. We demonstrated that procaine alone causes dose and time dependent growth inhibitory effect on NB4 cells.

Induction of apoptosis was observed after treatment with procaine alone. We didn't notice any expression of early differentiation marker CD11b after procaine treatment of NB4 cells.

In this study we investigated changes in expression of several genes (cadherin, G-CSF, PPARγ) that are important for NB4 cell differentiation.

Cadherins participate in cell differentiation. Different mechanisms for inactivating E-cadherin/CDH1 have been identified in human cancers. Promoter hypermethylation is an important epigenetic event associated with loss of E-cadherin gene expression during cancer progression. Few studies were published about reactivation of functional E-cadherin in certain cancer cell lines on treatment with the demethylating agent 5-aza-2′-deoxycytidine (5AzaC) [14-16]. We showed that procaine treatment can restore cadherin expression after 24 h of treatment.

G-CSF and its receptor are essential for basal and stress-induced granulopoiesis. The induction of the granulocytic differentiation of leukemic cells by ATRA has been a major breakthrough in terms of survival for acute promyelocytic leukaemia (APL) patients [17]. In our study we reported that DNMTI procaine alone did not induce G-CSF expression, but in the combined treatment with ATRA enhanced G-CSF expression compared with ATRA-treatment. This finding support our data, that procaine with ATRA induce granulocytic differentiation up to 76% at 72 h point.

We demonstrated that PPARγ gene expression can be induced after treatment with procaine. PPAR*y* activation by procaine correlated with inhibition of NB4 cell proliferation and induction of apoptosis.

Many of the changes in chromatin structure induced by transcription factors involve complex patterns of histone modifications by enzymes such as histone acetyltransferases (HATs), histone methyltransferases (HMTs), and kinases [18]. Histone modification strongly correlated with transcription activation in a wide variety of eukaryotic systems is H3 K4 methylation, especially the trimethylated state [19]. In this study we demonstrated that procaine caused H3 K4 trimethylation. These findings correlate with growth inhibitory and apoptotic effects of procaine on NB4 cells.

We also demonstrated induced histone acetylation including phospho-acetylation of histone H3 (S10/K9) after treatments with procaine or ATRA. This modification was detected in a smaller level after the combined treatment with procaine and ATRA in comparison with the treatment with procaine or ATRA alone. The decrease of the phospho-acetylation of histone H3 (S10/K9) coincide with the growth inhibition of leukemic cells after combined treatment. Phosphorylation particularly that of histones H1 and H3, has long been implicated in chromosome condensation during mitosis. However, converging evidence suggests that H3 phosphorylation (specifically serine 10) is also directly correlated with the induction of immediate-early genes such as c-jun, c-fos and c-myc. The potential importance of the serine 10 phosphorylation mark in H3 is strengthened by the finding that MSK1, a kinase activated by growth factor and stress stimuli, also phosphorylates H3 *in vitro*. H3 phosphorylation at serine 10 in conjunction with phosphorylation at serine 28, is also required for proper segregation and condensation of chromosomes during mitosis and meiosis [20-21].

We suggest that procaine can be used for further detailed investigations on epigenetic and differentiation therapy especially when used in combination with retinoic acid or similar agent. It can be promising agent for anticancer therapy.

5 Conclusions

Multiple genetic and epigenetic changes can cause the development of leukemic cells carrying unlimited self-renewal and drug-resistant properties for initiation of the leukaemia. New therapies designed to target leukaemia cells and leukaemia stem cells could eliminate cancer without the risks and side effects of current treatments that also destroy healthy cells in the body. Since the scientists still work on new directions for cancer stem cells therapy, it is necessary to learn more about cancer cell growth, apoptosis and differentiation mechanisms by using new drugs and combination of existing therapies. It could provide a cure in this case. The manuscript is dedicated on studies of new therapies using Procaine that target the leukemic cell population.

Conflict of interest: The authors report no potential conflicts of interest in this work and have nothing to disclose.

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