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Comparative effects of retinoic acid, vitamin D and resveratrol alone and in combination with adenosine analogues on methylation and expression of phosphatase and tensin homologue tumour suppressor gene in breast cancer cells

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Abstract

Aberrations in DNA methylation patterns have been reported to be involved in driving changes in the expression of numerous genes during carcinogenesis and have become promising targets for chemopreventive action of natural compounds. In the present study, we investigated the effects of all-*trans* retinoic acid (ATRA), vitamin D_3 and resveratrol alone and in combination with adenosine analogues, 2-chloro-2′-deoxyadenosine (2CdA) and 9-β-D-arabinosyl-2-fluoroadenine (F-ara-A), on the methylation and expression of phosphatase and tensin homologue (*PTEN*) tumour suppressor gene in MCF-7 and MDA-MB-231 breast cancer cells. The present results showed that in non-invasive MCF-7 cells, ATRA, vitamin D_3 and resveratrol possess high efficacy in the reduction of *PTEN* promoter methylation. It was associated with *PTEN* induction as well as DNA methyltransferase down-regulation and *p21* up-regulation after treatments with vitamin D_3 and resveratrol, suggesting a complex regulation of the DNA methylation machinery. Vitamin D_3 and resveratrol improved the inhibitory effects of 2CdA and F-ara-A on *PTEN* methylation in MCF-7 cells; however, only the combined action of vitamin D_3 and 2CdA boosted the induction of *PTEN* expression, suggesting a cooperation of these compounds in additional processes driving changes in *PTEN* expression. In contrast, in highly invasive MDA-MB-231 cells, only vitamin D_3 reduced *PTEN* methylation and induced its expression without notable effects in combined treatments. The present results suggest that natural compounds can find application in epigenetic anticancer therapy aimed at inhibition of promoter methylation of tumour suppressor genes and induction of their expression at early stages of carcinogenesis.

Key words: Vitamins: Phyto-oestrogens: Promoter methylation: Chemoprevention: Epigenetic therapy

Cancer initiation and progression require concurrent changes in the expression of multiple genes. While genetic alterations account for some of these changes, epigenetic modifications, such as aberrations in DNA methylation patterns, have attracted a significant amount of attention as a potential cause of concerted changes in the regulation of the expression of numerous genes during carcinogenesis[1–3]. The hallmarks of cancer cells are hypermethylation of certain DNA regions, such as promoters of tumour suppressor genes resulting in silencing of gene transcription, and global hypomethylation causing genomic instability[1,4]. Since aberrations in DNA methylation patterns are reversible and often observed at early stages of cancer development, it has become reasonable to consider them as targets for chemoprevention and anticancer therapy. Of particular interest is the inhibition of DNA methylation because a number of tumour suppressor genes have been reported to be transcriptionally silenced by promoter hypermethylation during carcinogenesis[5]. Hence, reactivation of tumour suppressor genes by a decrease in their promoter methylation may be a putative mechanism that contributes to repression of cancer growth and/or cancer initiation.

The present study was designed to elucidate the chemopreventive properties of all-*trans* retinoic acid (ATRA), vitamin D_3

Abbreviations: 2CdA, 2-chloro-2′-deoxyadenosine; 5-aza-dCyd, 5-aza-2′-deoxycytidine; AP-1, activator protein 1; ATRA, all-*trans* retinoic acid; *DNMT1*, DNA methyltransferase 1; ER, oestrogen receptor; F-ara-A, 9-β-D-arabinosyl-2-fluoroadenine; IC_{50}, inhibitory concentration; *PTEN*, phosphatase and tensin homologue; SAH, *S*-adenosyl-L-homocysteine; SAM, *S*-adenosyl-L-methionine.

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and resveratrol, focusing on their effects on the methylation and expression of phosphatase and tensin homologue (*PTEN*) tumour suppressor gene in MCF-7 and MDA-MB-231 breast cancer cells. The two cell lines differ with the steroid receptor status: MCF-7 cells are oestrogen receptor (ER)-positive, while MDA-MB-231 are ER-negative, which was previously linked to the differences in the invasiveness of breast cancer cells[6]. Choosing these cell lines as an experimental model allowed us to assess the effects of the tested compounds at the non-invasive (MCF-7) and invasive (MDA-MB-231) stages of breast cancer development.

PTEN, which was the subject of the experiments, was found to be silenced via hypermethylation in many types of human cancers, including breast cancer[7–10]. *PTEN* encodes a protein involved in the down-regulation of two main intracellular oncogenic signal transduction pathways, phosphoinositide 3-kinases/Akt and mitogen-activated protein kinase/activator protein 1 (AP-1)[11]. Since AP-1 transcription factor downstream of these pathways up-regulates DNA methyltransferase 1 (*DNMT1*) expression[12], it raises the possibility that activation of *PTEN* via demethylation induced by the tested compounds can reduce AP-1 activity and consequently restore normal regulation of *DNMT1* expression.

The basis for the studies was a premise that a growing body of literature demonstrates that ATRA, vitamin D_3 and resveratrol are capable of up-regulation of *p21* and *PTEN* expression, activation of retinoblastoma as well as inhibition of E2F and AP-1 transcription factor activities[13–18]. Since the above-mentioned targets for the natural compounds are indirectly or directly involved in the regulation of DNMT1 activity and *DNMT1* expression[12,19,20], our hypothesis is that these compounds may play an important role in the epigenetic regulation of gene expression.

Emerging evidence suggests that cancer-preventive agents might be combined with chemotherapy for improving the effectiveness of treatments. For instance, ATRA and vitamin D_3 were reported to improve the effect of 5-aza-2′-deoxycytidine (5-aza-dCyd) on growth inhibition and induction of differentiation in leukaemia and breast cancer cells[21,22]. These results prompted us to investigate whether ATRA, vitamin D_3 and resveratrol can be combined with adenosine analogues, 2-chloro-2′-deoxyadenosine (2CdA) and 9-β-D-arabinofuranosyl-2-fluoroadenine (F-ara-A), in order to enhance the reactivation of methylation-silenced tumour suppressor genes. 2CdA and F-ara-A, commonly used in the treatment of leukaemia, exert the cytotoxic effect mainly via inhibition of DNA synthesis by their phosphorylated derivatives[23]. However, our previous studies revealed that they inhibit *S*-adenosyl-L-homocysteine (SAH) hydrolase, an enzyme of the 'active methyl' cycle, and C-5 methyltransferase activities[24,25]. Moreover, our recent work demonstrated hypomethylation and induction of *RARbeta2* in breast cancer cells treated with the nucleoside analogues[26]. 5-aza-dCyd, a potent inhibitor of DNMT1, was used in our experiments as a reference agent since it was shown to reduce promoter methylation and activate transcription of tumour suppressor genes in numerous human cancers[27].

The present findings provide evidence that the tested natural compounds, ATRA, vitamin D_3 and resveratrol, possess high efficacy in the reduction of *PTEN* promoter methylation in non-invasive MCF-7 cells. These changes in DNA methylation are linked to induction of *PTEN* expression and are accompanied by down-regulation of *DNMT1* and up-regulation of *p21* after treatment with each compound, except for ATRA. In highly invasive MDA-MB-231 cells, only vitamin D_3 action results in *PTEN* hypomethylation and induction. Furthermore, we showed that vitamin D_3 and resveratrol considerably enhance the action of adenosine analogues on demethylation and/or induction of *PTEN*, however, only in MCF-7 cells. The results indicate that the action of the natural compounds is specific for the early non-invasive cancer stage, which may have profound implications for the prevention of carcinogenesis.

Materials and methods

Compounds

Nucleoside analogues, 2CdA, F-ara-A and 5-aza-dCyd, and natural compounds, ATRA, vitamin D_3 and resveratrol, were purchased from Sigma-Aldrich (St Louis, MO, USA). The tested nucleoside analogues were dissolved in water at the concentration of 1 mM and stored at −20°C. The natural compounds were dissolved in 96% ethanol at the concentration of 1 mM and stored in the dark at −20°C.

Cells and culture conditions

Human breast adenocarcinoma cell lines, MCF-7 and MDA-MB-231 (European Collection of Cell Cultures, Salisbury, UK), were cultured in Dulbecco's modified Eagle's medium (Cambrex Bio Science, Verviers, BE, Petit-Rechain, Belgium) and Leibovitz's L15 (Sigma-Aldrich) media, respectively, supplemented with 2 mM-glutamine (Sigma-Aldrich), 10% (and, for MDA-MB-231 cells, 15%) fetal bovine serum (Gibco, Scotland, UK), penicillin (1 U/ml) and streptomycin (1 μg/ml) (Gibco). Cells were grown in a humidified atmosphere of 5% CO_2 (except for the MDA-MB-231 cell line – without CO_2) at 37°C. Cells were routinely cultured in T-25 flasks in 10 ml of the standard medium and harvested by trypsinisation every 3–4 d after reaching 70–80% confluency. For the experiments with the tested compounds, MCF-7 and MDA-MB-231 cells were seeded at a low density of $2{\cdot}5 \times 10^4$ and 4×10^4/ml, respectively. After 24 h, the compounds were added in the culture medium and incubation was continued for 72 h.

Cytotoxicity assay

Cell viability was estimated by the trypan blue (Sigma-Aldrich) exclusion test as described previously[26], assuming that cells that stain as positive – because of disrupted membranes – are dead. The number of trypan blue-negative cells after 72 h treatment with the compounds alone or in combination is expressed as a percentage of non-staining cells in the culture without drugs (control, untreated cells). A concentration

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that caused a 50 % decrease in the number of non-staining cells after exposure to a compound when compared with untreated cells was referred to as the inhibitory concentration (IC_{50}). The number of trypan blue-positive cells after exposure to each compound is expressed as a percentage of the total cell number and referred to as the percentage of dead cells. All other experiments were performed at the concentrations of the tested compounds for which the percentage of dead cells lower than 10 % on day 3 was observed.

RNA extraction and complementary DNA synthesis

Total RNA was isolated using TRIZOL® (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. Isolated RNA was diluted in water containing 1 % diethylpyrocarbonate (a ribonuclease inhibitor; Sigma-Aldrich) and stored at −70°C. Total RNA (2 μg) was a template for complementary DNA synthesis. Briefly, 0·005 μg/μl of random hexamers (Promega, Madison, WI, USA) and 0·004 μg/μl of oligo(dT)$_{15}$ (Promega) were added to 2 μg of RNA in 50 μl of water. After incubation for 10 min at 70°C, the following chemicals were added: 20 μl of 5 × reaction buffer (250 mM-Tris–HCl, pH 8·3; 15 mM-$MgCl_2$; 375 mM-KCl; 50 mM-dithiothreitol), deoxyribonucleotide triphosphate mix (0·5 mM each; Promega), 3 mM-$MgCl_2$ and 2 μl of ImProm-II RT (Promega). Thermal cycling conditions were as follows: 5 min at 25°C, 60 min at 42°C and 15 min at 70°C.

Real-time PCR

For *PTEN*, *DNMT1* and *p21* expression analysis, primers were designed so that they overlapped splice junction, thereby avoiding the potential amplification of genomic DNA. The following primers were used: 5′-CGAACTGGTGTAATGATATGT-3′ (forward) and 5′-CATGAACTTGTCTTCCCGT-3′ (reverse) for *PTEN* (annealing temperature 50°C); 5′-ACCGCCCCTGGCCAAAGCCATTG-3′ (forward) and 5′-AGCAGCTTCCTCCTCCTTTATTTTAGCTGAG-3′ (reverse) for *DNMT1* (annealing temperature 60°C); 5′-GCTCAGGGGAGCAGGCTGAAG-3′ (forward) and 5′-CGGCGTTTGGAGTGGTAGAAATCTGT-3′ (reverse) for *p21* (annealing temperature 60°C). All PCR were carried out in a Rotor-Gene RG-3000 machine (Corbett Research, Cambridge, UK). The single reaction mixture contained 2 μl of 10 × PCR buffer (100 mM-Tris–HCl, pH 9·0; 500 mM-KCl; 1 % Triton X-100), 2 mM-$MgCl_2$ (Promega), deoxyribonucleotide triphosphate mix (200 μM each; Promega), forward and reverse primers (200 nM each; IBB, Warsaw, Poland), 1 μl of 20 × EvaGreen fluorescence dye (Biotium, Hayward, CA, USA), two units of Taq polymerase (Promega) and 1 μl of complementary DNA in a final volume of 20 μl. After an initial 2 min denaturation step at 94°C, PCR was carried out for fifty cycles at the following thermal conditions: 30 s at 94°C, 15 s at the annealing temperature and 30 s at 72°C. The relative expression of each tested gene was normalised to the geometric mean of four housekeeping genes, 40S ribosomal protein S17, 60S acidic ribosomal protein P0, H3 histone family 3A and β2-microglobulin, and calculated according to Pfaffl's method[28].

Methylation-sensitive restriction analysis

Methylation status of *PTEN* promoter was estimated using methylation-sensitive restriction analysis according to Iwase's method[29]. To normalise the results of evaluation of methylation and expression, DNA and RNA were isolated from the same sample after homogenisation of cells with Trizol. Genomic DNA was recovered from the organic phase according to the manufacturer's protocol after treatment of the cell pellet with Trizol and chloroform.

Genomic DNA (0·5 μg) was incubated with twenty units of HpaII (C$^{\downarrow}$CGG) (New England BioLabs, Hitchin, UK) restriction enzyme, 2 μl of restriction enzyme buffer and water in a total volume of 20 μl at 37°C overnight. Simultaneously, the control sample without enzyme and MspI-digested sample were incubated in the same conditions. Following incubation, control and digested DNA were amplified in PCR using the following primers for *PTEN*: 5′-GCGGAAGCAGCCGTTCGGAG-3′ (forward) and 5′-GTCATGTCTGGGAGCCTGTG-3′ (reverse; annealing temperature 60·9°C). The tested fragment of *PTEN* promoter was localised from −281 to +5 (GenBank accession no. AF143312) relative to the transcription start site (+1). This fragment encompassed one HpaII site near the binding sequence for AP-4 methylation-sensitive transcription factor.

In methylation-sensitive restriction analysis, the reaction mixture for PCR contained 2·5 μl of 10 × reaction buffer (100 mM-Tris–HCl, pH 8·9; 20 mM-$MgCl_2$; 500 mM-KCl), deoxyribonucleotide triphosphate mix (200 μM each; Promega), forward and reverse primers (1 μM each; IBB), 10 % dimethyl sulfoxide (Sigma-Aldrich), 1·25 units of Taq polymerase (Promega) and 0·1 μg of DNA in a final volume of 25 μl. The reaction was carried out in a Tpersonal Thermal Cycler (Biometra, Goettingen, Germany) at 95°C for 5 min, cycled at 1 min at 94°C, 1 min at the annealing temperature and 1 min at 70°C (twenty-six cycles), followed by a 5 min extension at 72°C. The amplified PCR products were fractionated on a 6 % polyacrylamide gel, stained with ethidium bromide and visualised under UV illumination. Band intensity was evaluated by densitometric analysis using QuantityOne software (Bio-Rad Laboratories Limited, Hemel Hempstead, Herts, UK). The methylation level in each sample was calculated on the basis of densitometric analysis and is expressed as a percentage of undigested DNA after the comparison of band intensities for digested and undigested DNA, which is as follows:

(Band intensity for DNA digested with HpaII/band intensity for undigested DNA) × 100%.

The percentage of methylation inhibition was calculated by comparison of the methylation level in control cells (untreated) and in cells treated with each compound, which is as follows:

100% − ((methylation level after treatment/methylation level in control) × 100%).

Statistical analysis

Data were assessed by one-way ANOVA followed by Tukey's *post hoc* test. Data are given as means and standard

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deviations of three to five independent experiments. The results were considered as statistically significant when $P<0{\cdot}05$.

Results

Promoter methylation and expression levels of phosphatase and tensin homologue in MCF-7 and MDA-MB-231 cells

The results indicated that *PTEN* promoter is methylated in 30% of MCF-7 cells and in almost 100% of MDA-MB-231 cells (Fig. 1(a)). Simultaneously, real-time PCR revealed that highly invasive MDA-MB-231 cells show a 30% lower *PTEN* expression and a 60% higher *DNMT1* mRNA level compared with non-invasive MCF-7 cells (Fig. 1(b)). Interestingly, higher *DNMT1* expression in MDA-MB-231 cells was accompanied by higher *PTEN* promoter methylation level and lower *PTEN* expression. It confirms an inverse correlation between promoter methylation and expression and suggests epigenetic regulation of *PTEN* transcription. Since differences in *PTEN* methylation and expression are observed between highly invasive MDA-MB-231 and non-invasive MCF-7 cells, *PTEN* methylation and expression levels may be considered as targets for clinical studies aimed at searching for biomarkers of breast cancer progression and/or invasiveness.

Tested drugs have varied effects on viability of MCF-7 and MDA-MB-231 cells

Regarding the trypan blue cytotoxicity assay as a measure of viability, it was observed that the tested compounds inhibited

(a)

Gene	Endonuclease	Cell line	Methylation status (%) Mean	SD
PTEN	HpaII	MCF-7	30·0	3·2
		MDA-MB-231	94·4	5·2

(b)

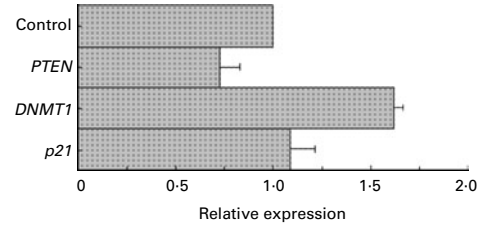


Fig. 1. Methylation and expression levels of phosphatase and tensin homologue (*PTEN*) in MCF-7 and MDA-MB-231 cells. (a) Methylation status of *PTEN* promoter in MCF-7 and MDA-MB-231 cells. Methylation level was estimated as described in Materials and methods. Values are means and standard deviations of five independent experiments. (b) Relative expression of *PTEN*, DNA methyltransferase 1 (*DNMT1*) and *p21* in MDA-MB-231 cells in comparison with MCF-7 cells. Expression level of each gene in MDA-MB-231 cells was compared with its expression in MCF-7 cells (control) and showed as a fold change. Values are means of three independent experiments, with standard deviations represented by horizontal bars.

(a)

Compound	IC_{50} value (μM) MCF-7	MDA-MB-231
ATRA	0·35	Not reached
Vitamin D_3	1·5	2·5 (25·0)
RES	14·0	12·0
2CdA	0·2	0·2
F-ara-A	15·0	4·0
5-aza-dCyd	0·6	4·0

(b)

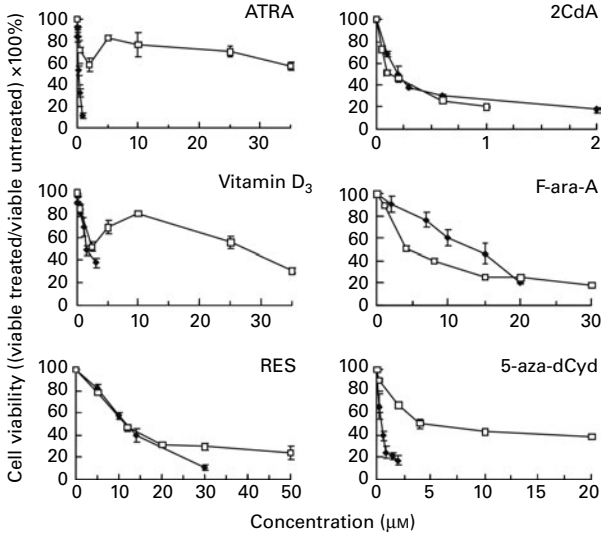


Fig. 2. Effects of natural compounds and nucleoside analogues on MCF-7 and MDA-MD-231 cell viability compared with untreated cells (control). (a) Concentrations of nucleoside analogues and natural compounds causing a 50% inhibitory effect on cell viability when compared with untreated cells (IC_{50} value). (b) Inhibitory effect of the natural compounds and nucleoside analogues on cell viability in the MCF-7 (◆) and MDA-MB-231 (□) cell lines. Values are means of four independent experiments, with standard deviations represented by vertical bars. ATRA, all-*trans* retinoic acid; RES, resveratrol; 2CdA, 2-chloro-2′-deoxyadenosine; F-ara-A, 9-β-D-arabinosyl-2-fluoroadenine; 5-aza-dCyd, 5-aza-2′-deoxycytidine.

MCF-7 and MDA-MB-231 cell viability when compared with untreated cells in a dose-dependent manner, apart from ATRA and vitamin D_3 in MDA-MB-231 cells. After 72 h culture, concentrations causing a 50% decrease in the number of viable cells after exposure when compared with untreated cells (IC_{50}) were determined (Fig. 2). In the case of resveratrol and 2CdA, IC_{50} values were similar for both cell lines, contrary to ATRA and vitamin D_3 which affected MCF-7 cell viability at lower doses than estimated for MDA-MB-231 cells (Fig. 2). ATRA reduced viability of MDA-MB-231 cells only by 30% and the highest suppression was caused by 2 and 25 μM-ATRA. For vitamin D_3, two IC_{50} values, 2·5 and 25 μM, were determined in invasive cells and a dose-dependent manner of action was observed at concentrations higher than 10 μM. Similarly, after treatment with F-ara-A, a 50% decrease in MDA-MB-231 cell viability was reached at a 4-fold lower concentration compared with MCF-7 cells. We assumed that inhibitory effects of ATRA, vitamin D_3 and

F-ara-A on cell viability are dependent on invasiveness of breast cancer. The contrary effect was observed for 5-aza-dCyd where inhibition of MDA-MB-231 cell viability required a 7-fold higher concentration than for MCF-7 cells, which may be associated with a 60% higher expression of *DNMT1* in invasive cells.

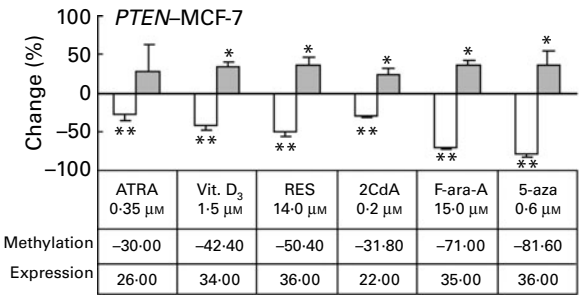
The percentage of dead cells after challenge with both natural compounds and nucleoside analogues was much higher in non-invasive MCF-7 cells than in invasive MDA-MB-231 cells after 72 h culture (data not shown). For instance, the percentage of dead cells after treatment with resveratrol at 30 μM was equal to 24% for MCF-7 and 4% for MDA-MB-231 cells. After exposure to 0·5 μM-ATRA, the number of cells stained by trypan blue was 14% for MCF-7 and only 2% for MDA-MB-231 cells. The results suggest that invasive breast cancer cells at an advanced stage of progression are less sensitive to the tested compounds, although a mechanism underlying this observation remains to be elucidated.

Tested drugs at inhibitory concentrations have a profound effect on phosphatase and tensin homologue promoter methylation and expression only in the MCF-7 cell line

MCF-7 cells. We revealed a strong inhibitory effect of both natural compounds and nucleoside analogues on *PTEN* promoter methylation when MCF-7 cells were treated for 72 h using IC_{50} concentrations (Fig. 3(a)). Among the natural compounds, only vitamin D_3 and resveratrol led to a 35% increase in *PTEN* expression, which was associated with a 50% reduction of promoter methylation. In cells exposed to F-ara-A or 5-aza-dCyd, gene expression was also up-regulated by 35% and promoter methylation was inhibited by 70–80%. Interestingly, the overall effect of vitamin D_3 and resveratrol was similar to the effect exerted by nucleoside analogues, including 5-aza-dCyd – a direct potent inhibitor of DNA methylation.

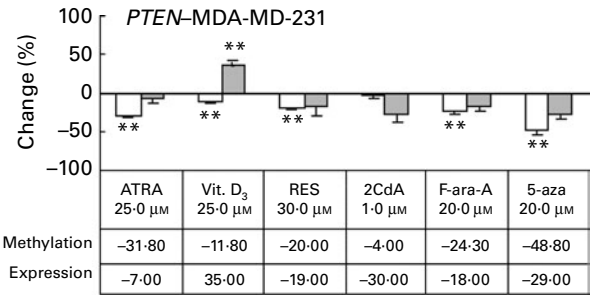
MDA-MB-231 cells. In MDA-MB-231 cells, all compounds, except for 5-aza-dCyd, had no significant influence on *PTEN* promoter methylation and expression when IC_{50}

(a)



	ATRA 0·35 μM	Vit. D_3 1·5 μM	RES 14·0 μM	2CdA 0·2 μM	F-ara-A 15·0 μM	5-aza 0·6 μM
Methylation	–30·00	–42·40	–50·40	–31·80	–71·00	–81·60
Expression	26·00	34·00	36·00	22·00	35·00	36·00

(b)



	ATRA 25·0 μM	Vit. D_3 25·0 μM	RES 30·0 μM	2CdA 1·0 μM	F-ara-A 20·0 μM	5-aza 20·0 μM
Methylation	–31·80	–11·80	–20·00	–4·00	–24·30	–48·80
Expression	–7·00	35·00	–19·00	–30·00	–18·00	–29·00

(c)

	MCF-7				MDA-MB-231			
	Relative expression				Relative expression			
	DNMT1		*p21*		*DNMT1*		*p21*	
Compound	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	1·0	0·0	1·0	0·0	1·0	0·0	1·0	0·0
ATRA	1·14	0·23	0·91	0·16	0·86	0·03	1·45	0·25
Vit. D_3	0·86*	0·11	1·78**	0·32	0·84	0·04	1·83*	0·11
RES	0·44**	0·02	1·22*	0·06	0·95	0·29	2·86**	1·06
2CdA	0·79*	0·11	1·46*	0·15	1·13	0·13	3·60**	0·78
F-ara-A	0·58**	0·10	3·23**	0·31	1·04	0·21	4·34**	1·02
5-aza	0·79*	0·04	2·61**	0·22	1·03	0·13	1·28	0·12

Fig. 3. Effects of the natural compounds and nucleoside analogues on phosphatase and tensin homologue (*PTEN*) promoter methylation (□) as well as *PTEN*, DNA methyltransferase 1 (*DNMT1*) and *p21* expression (▩) in MCF-7 and MDA-MB-231 cells. Changes in *PTEN* methylation and expression levels in (a) MCF-7 and (b) MDA-MB-231 cells. (c) *DNMT1* and *p21* relative expression levels in MCF-7 and MDA-MB-231 cells. DNA methylation changes and relative expression were calculated as described in Materials and methods. Expression data for *DNMT1* were extracted from our previous work[26]. Values are means of three to five independent experiments, with standard deviations represented by vertical bars. Statistical analyses were performed by ANOVA followed by Tukey's *post hoc* test. Mean values of treatment were significantly different from those of control: $^*P<0·05$, $^{**}P<0·01$. ATRA, all-*trans* retinoic acid; Vit. D_3, vitamin D_3; RES, resveratrol; 2CdA, 2-chloro-2′-deoxyadenosine; F-ara-A, 9-β-D-arabinosyl-2-fluoroadenine; 5-aza-dCyd, 5-aza-2′-deoxycytidine.

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concentrations were used (data not shown). The 72 h treatment with doses several fold higher than IC_{50} (shown in Fig. 3(b)) resulted in a significant reduction of *PTEN* promoter methylation, the highest being approximately 30–50 % for ATRA and 5-aza-dCyd (Fig. 3(b)). Interestingly, an effect on gene expression was only exerted by vitamin D_3, which led to a 35 % increase in *PTEN* mRNA level. Other compounds did not influence *PTEN* expression despite reduction of promoter methylation (Fig. 3(b)). It suggests that not only promoter methylation but also other aberrations, for example histone modifications, are implicated in the regulation of *PTEN* transcription in MDA-MB-231 cells, and vitamin D_3 action may be sufficient to up-regulate *PTEN* expression owing to the regulation of these modifications. It has previously been shown that vitamin D_3 may regulate gene transcription through modifications of histone deacetylation and histone demethylation[30].

Changes in phosphatase and tensin homologue methylation and expression levels are accompanied by p21 *induction and DNA methyltransferase 1 reduction in the MCF-7 cell line*

MCF-7 cells. The inverse correlation between *p21* and *DNMT1* expression was previously revealed in both normal and cancer cells. An increase in the expression of *p21* can influence *DNMT1* activity/expression owing to competition of p21 and DNMT1 for the same binding site on the proliferating cell nuclear antigen[31,32]. In MCF-7 cells, the inverse correlation between *p21* and *DNMT1* expression was observed after treatments with vitamin D_3 and resveratrol as well as with all tested nucleoside analogues, with the strongest effect exerted by F-ara-A (a 3-fold increase in *p21* and a 42 % decrease in *DNMT1*; Fig. 3(c)). Although vitamin D_3 led to an 80 % increase in *p21* mRNA level, only a 14 % decrease in *DNMT1* expression was observed. Resveratrol, in turn, led to a high decrease in *DNMT1* expression (56 %) despite only

Compounds	Concentration (μM)	Inhibition of *PTEN* methylation (%)	Relative expression *PTEN* Mean	SD	*DNMT1* Mean	SD	*p21* Mean	SD
Control	–		1·0	0·0	1·0	0·0	1·0	0·0
ATRA	0·3		1·39	0·25	1·32	0·38	0·69	0·10
2CdA	0·2		1·20*	0·09	0·79*	0·09	1·46**	0·12
2CdA + ATRA	0·2 + 0·3		0·81	0·12	0·66†	0·11	0·82	0·14
F-ara-A	10·0		1·25*	0·08	0·69*	0·19	1·15	0·08
F-ara-A + ATRA	10·0 + 0·3		0·80	0·10	0·52††	0·11	0·51	0·06
5-aza-dCyd	0·2		1·19	0·16	0·91	0·18	2·06**	0·50
5-aza-dCyd + ATRA	0·2 + 0·3		0·74	0·13	0·53†	0·15	1·24	0·21
Vit. D_3	1·5		1·34**	0·04	0·86	0·09	1·78**	0·26
2CdA	0·2		1·20*	0·09	0·79*	0·09	1·46**	0·12
2CdA + Vit. D_3	0·2 + 1·5		1·54**†††‡	0·09	0·56**†‡	0·10	1·65**	0·30
F-ara-A	10·0		1·25*	0·08	0·69*	0·19	1·15	0·08
F-ara-A + Vit. D_3	10·0 + 1·5		1·20**	0·05	0·64*	0·07	1·80**‡‡	0·30
5-aza-dCyd	0·2		1·19	0·16	0·91	0·18	2·06**	0·50
5-aza-dCyd + Vit. D_3	0·2 + 1·5		0·88	0·17	0·61*‡	0·14	2·12*	0·42
RES	10·0		1·30*	0·16	0·49**	0·09	1·15	0·08
2CdA	0·2		1·20*	0·09	0·79*	0·09	1·46**	0·12
2CdA + RES	0·2 + 10·0		0·84	0·14	0·78*	0·08	3·89**†††‡	0·43
F-ara-A	10·0		1·25*	0·08	0·69*	0·19	1·15	0·08
F-ara-A + RES	10·0 + 10·0		1·08	0·22	0·61	0·34	4·48**†††‡	0·23
5-aza-dCyd	0·2		1·19	0·16	0·91*	0·18	2·06**	0·50
5-aza-dCyd + RES	0·2 + 10·0		0·84	0·23	0·50*	0·31	2·44**††	0·52

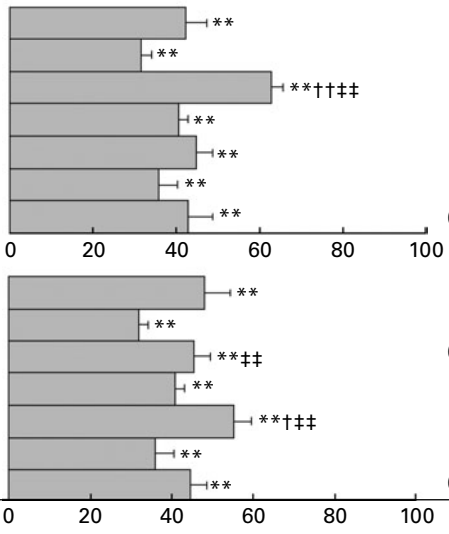

Fig. 4. Effects of a concurrent action of the natural compounds and nucleoside analogues on phosphatase and tensin homologue (*PTEN*) methylation and expression as well as on DNA methyltransferase 1 (*DNMT1*) and *p21* expression in MCF-7 cells. The percentage of methylation inhibition and the relative expression were calculated as described in Materials and methods. Expression data for *DNMT1* were extracted from our previous work[26]. Values are means of three independent experiments, with standard deviations represented by horizontal bars. Statistical analyses were performed by ANOVA followed by Tukey's *post hoc* test. Mean values of treatment were significantly different from those of control: *$P<0·05$, **$P<0·01$. Mean values of the natural compounds were significantly different from those of the combined treatment: †$P<0·05$, ††$P<0·01$. Mean values of the analogues were significantly different from those of the combined treatment: ‡$P<0·05$, ‡‡$P<0·01$. ATRA, all-*trans* retinoic acid; 2CdA, 2-chloro-2′-deoxyadenosine; F-ara-A, 9-β-D-arabinosyl-2-fluoroadenine; 5-aza-dCyd, 5-aza-2′-deoxycytidine; Vit. D_3, vitamin D_3; RES, resveratrol.

a 20% rise in *p21* expression. It suggests that there is an additional mechanism of resveratrol action on *DNMT1* regulation. It has recently been established that treatment of cancer cells with resveratrol decreases the levels of oncogenic microRNA, targeting, for example, *PTEN*, and increases the levels of tumour suppressor microRNA[33]. It raises an intriguing possibility that resveratrol may affect *DNMT1* expression by regulating microRNA, which remains to be elucidated.

MDA-MB-231 cells. In MDA-MB-231 cells, the tested compounds affected *p21* expression (Fig. 3(c)). The greatest effect with a 3- to 4-fold increase was exerted by resveratrol as well as by 2CdA and F-ara-A. However, we did not observe any strong correlation between *p21* up-regulation and *DNMT1* down-regulation after the 72 h treatments.

Combined treatment with vitamin D_3 or resveratrol and nucleoside analogues is efficient at non-invasive stages of breast cancer development

MCF-7 cells. In MCF-7 cells, the tested compounds were used in combined treatment at concentrations lower or equal to IC_{50} (values shown in Fig. 4) so that the percentage of dead cells was lower than 30%. The combined action of almost all natural compounds and nucleoside analogues resulted in enhancement of the inhibitory effects of the analogues on *PTEN* promoter methylation with significant changes for vitamin D_3 and 2CdA, ATRA and 5-aza-dCyd, resveratrol and 5-aza-dCyd (Fig. 4). Interestingly, the combined treatment with vitamin D_3 and 2CdA also contributed to a stronger induction of *PTEN* expression (28% additional increase) accompanied by a stronger reduction of *DNMT1* mRNA level compared with the analogue action alone (Fig. 4). Furthermore, the combined action of vitamin D_3 or resveratrol with adenosine analogues, 2CdA or F-ara-A, caused an additional increase in *p21* expression (Fig. 4). The most relevant improvement was demonstrated for concurrent action of resveratrol and 2CdA or resveratrol and F-ara-A, causing an additional increase in *p21* expression by 2·5- to 4-fold. As shown in Fig. 5, all the tested natural compounds and nucleoside analogues in combinations caused a relevant decrease in cell viability compared with single treatments despite the fact that not all combinations boosted the inhibition of *PTEN* promoter methylation and induction of *PTEN* expression. It suggests that apart from epigenetic reactivation of *PTEN* tumour suppressor gene, other mechanisms exist that the compounds cooperate in and that can contribute to the effect on cell viability. For instance, vitamin D_3 and 2CdA were shown to cooperate in the activation of the mitochondrial Ca^{2+}-mediated apoptotic pathway which can affect the cell number[34,35].

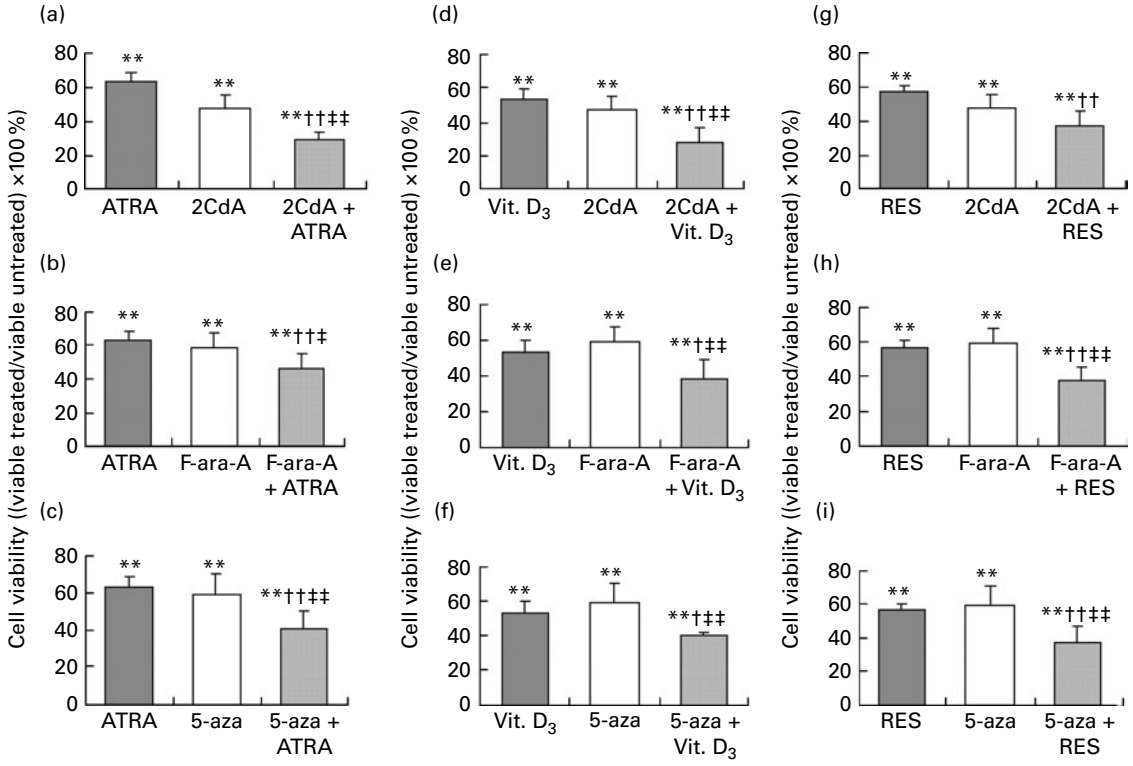

Fig. 5. Inhibitory effect of the natural compounds and nucleoside analogues in combination on the viability of MCF-7 cells when compared with untreated cells (control). The compounds were used at concentrations shown in Fig. 4. (a) All-*trans* retinoic acid (ATRA) + 2-chloro-2′-deoxyadenosine (2CdA), (b) ATRA + 9-β-D-arabinosyl-2-fluoroadenine (F-ara-A), (c) ATRA + 5-aza-2′-deoxycytidine (5-aza-dCyd), (d) vitamin D_3 (Vit. D_3) + 2CdA, (e) Vit. D_3 + F-ara-A, (f) Vit. D_3 + 5-aza-dCyd, (g) resveratrol (RES) + 2CdA, (h) RES + F-ara-A and (i) RES + 5-aza-dCyd. Values are means of four independent experiments, with standard deviations represented by vertical bars. Statistical analyses were performed by ANOVA followed by Tukey's *post hoc* test. Mean values of treatment were significantly different from those of control: *$P<0·05$, **$P<0·01$. Mean values of the natural compounds were significantly different from those of the combined treatment: †$P<0·05$, ††$P<0·01$. Mean values of the analogues were significantly different from those of the combined treatment: ‡$P<0·05$, ‡‡$P<0·01$.

MDA-MB-231 cells. In the combined treatment of MDA-MB-231 cells, the tested agents were used at concentrations higher than IC_{50} (values shown in Fig. 3(b)) since we did not observe significant effects at lower doses (values shown in Fig. 2(a)). The experiments revealed that combinations of the natural compounds and nucleoside analogues do not cause an additional decrease in cell viability at a highly invasive cancer stage (data not shown). The inhibitory effect of 5-aza-dCyd on *PTEN* promoter methylation after the combined treatment with ATRA or resveratrol was lower compared with the analogue action alone. A slight enhancement of *PTEN* methylation inhibition was observed after exposure to ATRA or resveratrol in combination with 2CdA or F-ara-A. Only the combined action of vitamin D_3 and 2CdA led to the reduction of *PTEN* methylation in a synergistic manner. However, it was accompanied neither by the induction of *PTEN* expression nor by the suppression of *DNMT1*.

Discussion

The present findings unravel a role of three natural compounds, ATRA, vitamin D_3 and resveratrol, in the regulation of DNA methylation, which can be considered as one of the mechanisms of their inhibitory action on cancer cell growth. In non-invasive ER-positive MCF-7 cells, all natural compounds caused reduction of *PTEN* promoter methylation, and vitamin D_3 and resveratrol also led to an increase in gene expression (Fig. 3(a)). In addition, the actions of vitamin D_3 and resveratrol resulted in a decrease in *DNMT1* and an increase in *p21* expression (Fig. 3(c)). In ER-negative MDA-MB-231 cells characterised by aggressive invasive capacity, the natural compounds led to a 12–32% reduction of *PTEN* promoter methylation, however, only when used at concentrations higher than IC_{50} (Fig. 3(b)). It was associated with an increase in *PTEN* mRNA level only in the presence of vitamin D_3. Our hypothesis is that demethylation and reactivation of methylation-silenced tumour suppressor genes by the tested natural chemicals might result from their involvement in the down-regulation of DNMT1 enzymatic activity and/or *DNMT1* expression. This indirect effect on DNMT1 activity seems to be active at the early stages of breast cancer development and can be attributed to the capability of the vitamins and phyto-oestrogens to stimulate *p21* and *PTEN* expression. As mentioned previously, p21 competes with DNMT1 for the same binding site on the proliferating cell nuclear antigen, which negatively regulates DNMT1 activity and subsequently expression[31,32]. PTEN is a negative regulator of AP-1 transcription factor activating *DNMT1* expression[12]. Therefore, *PTEN* activation by natural compounds may down-regulate AP-1-responsive genes, including *DNMT1* [36,37].

The diversity of the effects of the tested natural compounds in both cell lines may be partially explained by their ER status associated with their invasiveness[6]. Previous studies in human breast cancer cells indicated that ER stimulate activity of c-Ha-ras, which, in turn, leads to induction of the Ras/Raf/mitogen-activated protein kinase/AP-1 signalling pathway[38]. Since AP-1 transcription complex activates *DNMT1* promoter[12], inactivation of the ER may cause inhibition of AP-1 and, in consequence, reduction of *DNMT1* expression. ATRA and vitamin D_3 were shown to down-regulate ER abundance and function in MCF-7 cells, which might explain their stronger effects in this cell line compared with ER-negative MDA-MB-231 cells as we observe in the present study[39,40]. Similarly, resveratrol as a phyto-oestrogen interacts with ER which mediates, at least partially, its anticancer activities[41]. Resveratrol binds to ER and may act as a mixed agonist/antagonist depending on ER subtype and the sequence of ER response elements within the oestrogen-responsive genes[41]. Diverse effects exerted by resveratrol in the tested breast cancer cell lines can be associated with the fact that MCF-7 cells express ERα and ERβ while MDA-MB-231 cells express only low levels of ERβ[42].

It is noteworthy that challenge of MCF-7 cells with ATRA did not increase *PTEN* expression significantly (Fig. 3(a)). We suggest that a 30% decrease in promoter methylation after ATRA is not sufficient to reactivate *PTEN* and higher doses of the compound may be needed. On the other hand, the activation of *PTEN* after treatments with vitamin D_3 and resveratrol may result from the epigenetic regulation as well as other additional mechanisms as was shown for resveratrol activating *PTEN* through suppression of oncogenic microRNA[33].

Interestingly, the effects of the natural compounds, especially vitamin D_3 and resveratrol, on *PTEN* expression and to some extent on *PTEN* promoter methylation in MCF-7 cells were as profound as those caused by adenosine analogues (Fig. 3(a)). The present results indicate that 2CdA and F-ara-A are also capable of reduction of *PTEN* promoter methylation and activation of its transcription, although their mechanism of action seems to be different from that in the case of the natural chemicals. Adenosine analogues may affect DNA methylation state probably by inhibition of SAH hydrolase activity, which interferes with the 'active methyl' cycle. Previous studies[24,43] have shown that these adenosine analogues inhibit the activity of SAH hydrolase. Since this enzyme is crucial for a breakdown of SAH to adenosine and homocysteine, its inhibition leads to subsequent accumulation of SAH, which is a metabolite of a methyl donor *S*-adenosyl-L-methionine (SAM) formed after demethylation of SAM as well as a powerful inhibitor of methyltransferases owing to competition for the SAM binding site[25]. An increase in a concentration of SAH results in a decrease in SAM level and inhibition of methylation of many tissue components, including DNA, RNA, proteins and phospholipids, which led to liver cancer development in rats fed with a SAM-deficient diet[44].

In the studies undertaken, our attention was also focused on a question whether the natural compounds are able to enhance the effects of nucleoside analogues on cell viability, DNA methylation and expression of tumour suppressor genes. It has previously been reported that in MCF-7 cells, treatment with 5-aza-dCyd, valproic acid (histone deacetylase inhibitor) and ATRA led to the activation of *RARβ*[22]. In a few breast cancer cell lines, including MCF-7, an ATRA-mediated increase in *RARβ* expression was visible after pre-treatment of cells with 5-aza-dCyd[45]. Furthermore, combination of 5-aza-dCyd with ATRA or vitamin D_3 resulted in the demethylation and

activation of *p16* in leukaemic cells[21]. The present results revealed a 70 % decrease in the methylation of *PTEN* promoter after the combined treatment of MCF-7 cells with 5-aza-dCyd and ATRA, which was about 40 % more than in cells treated with these compounds alone (Fig. 4). This robust combined effect may be explained by a strong, almost 50 %, down-regulation of *DNMT1*. Despite changes in methylation, we did not observe *PTEN* re-expression for this drug combination, even though it has been reported that the complex of retinoic acid with receptors and co-activators from the p160 and p300 family is able to activate histone acetyltransferase which changes chromatin structure to be easily accessible for proteins of transcription complex[46]. Similarly, the treatments with vitamin D_3 and 5-aza-dCyd as well as with resveratrol and 5-aza-dCyd did not affect *PTEN* expression (Fig. 4). Although all natural compounds improved the inhibitory effects of 2CdA and F-ara-A on *PTEN* methylation, only the combined action of vitamin D_3 and 2CdA boosted the induction of *PTEN* expression (Fig. 4). It suggests that these compounds may cooperate in other unknown mechanisms driving changes in *PTEN* expression.

The present data suggest that the tested natural compounds, most notably vitamin D_3 and resveratrol, can become promising agents in epigenetic therapy aimed at inhibition of promoter methylation of tumour suppressor genes and induction of their expression at the non-invasive stages of cancer development. Since aberrations in DNA methylation patterns are frequently detected during initiation of carcinogenesis, protection against these changes may be relevant to chemoprevention. However, the potential ability of the tested natural compounds to protect from alterations in DNA methylation and from cancer needs to be elucidated in future experiments. The present results also indicate that the natural compounds may improve the effectiveness of chemotherapy at the non-invasive cancer stage since they enhanced the effects of nucleoside analogues on the reduction of cell viability and/or on the stimulation of the expression of *PTEN* tumour suppressor gene in non-invasive MCF-7 cells.

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