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# Condurango 30C Induces Epigenetic Modification of Lung Cancer-specific Tumour Suppressor Genes via Demethylation

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### Keywords

DNA methylation · CpG islands ·  
Epigenetic modification · Condurango 30C ·  
Lung cancer · PCR-SSCP analysis · Homeopathy

### Schlüsselwörter

DNA-Methylierung · CpG-Inseln ·  
Epigenetische Modifikation · Condurango 30C ·  
Lungenkrebs · PCR-SSCP-Analyse · Homöopathie

### Summary

**Background:** DNA hypermethylation induces cancer progression involving CpG island of DNA and causes inactivation of tumour suppressor genes. In this study, DNA hypermethylation status of lung cancer and ability of ultra-highly diluted Condurango 30C to modulate DNA methylation were ascertained by analysis of lung cancer-specific tumour suppressor genes in respect to placebo. **Materials and Methods:** DNA methylation status, if any, was determined by PCR-SSCP analyses in lung cancer-specific tumour suppressor genes (p15, p16 and p53) using H460-NSCLC cell and BaP-induced lung cancer of rats. The ability of Condurango 30C to modulate DNA methylation, if any, was verified against placebo control in blinded manner. **Results:** Condurango 30C-treated DNA showed significant decrease in band intensity of p15 and p53 genes especially in methylated condition in vitro, at IC<sub>50</sub> dose (2.43 µl/100 µl). SSCP analysis of p15 and p53 genes in Condurango 30C-treated DNA also suggests that Condurango 30C can decrease methylation, in vitro. Inhibition of p15 hypermethylation was observed in post-cancer treatment of rats with Condurango 30C. SSCP results gave a better indication of differences in band position of p15 and p53 in Condurango 30C-treated lung samples. **Conclusion:** Condurango 30C could trigger epigenetic modification in lung cancer via modulation of DNA hypermethylation.

### Zusammenfassung

**Hintergrund:** DNA-Hypermethylierung verursacht unter Einbezug von CpG-Inseln der DNA die Progression von Krebs und führt zur Inaktivierung von Tumorsuppressorgenen. In der vorliegenden Studie wurde der DNA-Hypermethylierungsstatus bei Lungenkrebs untersucht sowie die Fähigkeit von ultrahoch verdünntem Condurango 30C, DNA-Methylierung zu beeinflussen. Die Untersuchung erfolgte über eine Analyse von Lungenkrebs-spezifischen Tumorsuppressorgenen in Vergleich zu Placebo. **Material und Methoden:** Der DNA-Methylierungsstatus wurde, falls vorhanden, in den Lungenkrebs-spezifischen Tumorsuppressorgenen (p15, p16 und p53) mithilfe von H460-NSCLC-Zellen und BaP-induziertem Lungenkrebs bei Ratten über PCR-SSCP-Analysen bestimmt. Die Fähigkeit von Condurango 30C, eine eventuelle DNA-Methylierung zu beeinflussen, wurde verblindet mit einer Placebo-Gruppe verglichen. **Ergebnisse:** Mit Condurango 30C behandelte DNA zeigte einen signifikante Schwächung der Band-Intensität von p15- und p53-Genen, insbesondere in methyliertem Zustand in-vitro und bei einer Dosis von IC<sub>50</sub> (2.43/100 µl). Die Ergebnisse der SSCP-Analyse von p15- und p53-Genen in der mit Condurango 30C behandelten DNA sprechen zudem für die Fähigkeit von Condurango 30C, die Methylierung in-vitro zu verringern. Die Unterdrückung der p15-Hypermethylierung wurde in der Krebsnachbehandlung von Ratten mit Condurango 30C beobachtet. In der SSCP-Analyse zeigten sich die Unterschiede in den Band-Positionen von p15 und p53 deutlicher, wenn die Lungenproben mit Condurango 30C behandelt wurden. **Conclusion:** Condurango 30C könnte über die Modulation von DNA-Hypermethylierung eine epigenetische Modifikation bei Lungenkrebs auslösen.

## Introduction

DNA hypermethylation is an important aspect involved in carcinogenesis and cancer progression that causes inactivation of tumour suppressor genes. A major form of epigenetic information in mammals is carried by DNA methylation, represented by 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (hmC). In the last decade, aberrations in DNA methylation patterns have been found to be a common feature of human cancer [1]. CpG islands located in the promoter region of tumour suppressor genes, normally unmethylated at these regions like in all other genes, undergo a dense hypermethylation in cancer cells leading to gene silencing. In recent years, an increasing number of gene at CpG islands aberrantly hypermethylated in cancer have been extensively mapped [2].

In case of lung cancer, consumption of BaP through tobacco smoking is the important inducer for DNA hypermethylation. A plethora of studies described existence of DNA methylation in non-small cell lung cancer (NSCLC) [3]. Various tumour suppressor genes involved specifically in NSCLC, such as p15, p16 and p53, play a pivotal role in hypo- and hypermethylation-related studies. The crucial question is to ascertain whether hypermethylation of proto-oncogenes or hypermethylation of growth suppressor genes provides a selective advantage to cancer cells and therefore may represent a sequence of events leading to malignancy. Alternatively, the changes in methylation patterns may merely reflect the altered metabolism or physiology of rapidly dividing NSCLC cells [4].

Utilization of epigenetic regulatory mechanisms in disease states can contribute to the molecular pathology by altering gene expression states that support progression and inhibit defense pathways such as tumour suppressor genes. Hypermethylation of short and long interspersed repetitive elements has been reported in cancer [5]. So, there is a need to search for an alternative drug or agent which has the ability to modulate DNA-methylation, thus signifying its ability to modulate gene expression.

In homeopathy, the most controversial aspect is the use of ultra-high dilutions. Certain remedies are diluted beyond Avogadro's number ( $6 \times 10^{-23}$ ) and at this stage the original substance gets diluted out [6]. At potency 12C the dilution becomes  $10^{-24}$  which crosses the Avogadro's number and therefore, at potency 12C and higher (like 30C), the physical existence of even one single molecule of the original drug substance is highly improbable [7]. Yet, in clinical trials these ultra-highly diluted remedies demonstrate great ability to enhance recovery process by reducing disease symptoms [8]. Nanoparticles of drug are claimed to be still present in such ultra-high dilutions, which may be attributed to the ability of the remedy to modify the structural orientation, size and physico-chemical properties of the resultant homeopathic drugs even in the absence of any original molecule [9–11]. Thus, homeopathy is no longer considered a myth, but needs active research to understand the complete mechanism of action of the ultra-high dilutions [12, 13] and to reinforce confidence in the use of this controversial system of medication. In this context, the gene regulatory hypothesis proposed by Khuda-Buksh [14, 15] that suggests the ability of the ultra-highly diluted remedies to trigger modulation of gene expres-

sion as a possible molecular mechanism in all living organisms has now been gaining ground with the accumulation of relevant research data in its support.

*Marsdenia condurango* (commonly called Condurango), used for treatment of certain cancer types, has already been demonstrated to have apoptosis-inducing potential in its different forms (ethanolic extract, glycoside-rich components and homeopathic potentised forms [16–19]). The gene regulatory hypothesis is also supported by our recently published data on epigenetic modification via acetylation and deacetylation in HeLa cells after treatment with Condurango 30C [20]. These findings encouraged us to prove i) DNA-hypermethylation status of lung cancer-specific tumour suppressor genes (p15, p16 and p53); ii) if there is any modulation of hypermethylation status in DNA profiles of p15, p16 and p53 genes, in vitro and in vivo, after administration of Condurango 30C; and iii) if homeopathically diluted placebo (succussed 70% alcohol) could have a similar effect.

## Materials and Methods

### *In Vitro Study*

#### *Cell Culture and Drug Used*

NCI-H460 (H460), human NSCLC cells, were procured from National Centre for Cell Science (NCCS), Pune, India, and cultured in RPMI-1640 media, supplemented with 10% FBS and 1% antibiotic antimycotic solution. Homeopathic potentised drug Condurango 30C and suitable placebo (vehicle) with the same stock of alcohol (ethanol) were procured from Schwabe India Pvt. Ltd., Kolkata, and used in liquid form.

#### *Determination of Cell Viability*

H460 cells ( $1 \times 10^4$ /well) were plated in 96 well plates and treated with different concentrations of Condurango 30C (0.5  $\mu$ l /100 $\mu$ l media – 5  $\mu$ l /100 $\mu$ l media) and placebo (succussed 70% alcohol, drug vehicle) (0.5  $\mu$ l /100 $\mu$ l media – 5  $\mu$ l /100 $\mu$ l media) as control for 24h and 48h, respectively, from the stock solutions. Negative control (untreated) group received no drugs. The concentration at which the drug showed nearly 50% ( $IC_{50}$  value) cell death was determined by MTT assay [19].

#### *Genomic DNA Isolation*

$2 \times 10^6$  cells (untreated, placebo and drug-treated cells) were taken in 1.5 ml Eppendorf tubes, washed with 1x PBS and dissolved in 100 $\mu$ l DNA extraction buffer. DNA was extracted using conventional phenol/chloroform method [20]. 100 $\mu$ l 1x Tris-EDTA (TE) buffer was added to DNA pellet and mixed well by vortexing.

#### *Bisulfite Modification*

The optical density (O.D.) of isolated DNA was taken spectrophotometrically at 260 nm. 1  $\mu$ g DNA from each experimental set-up was taken for bisulfite modification. DNA was taken in sterilised PCR tubes and heated at 100°C for 10 min. DNA samples were quick-chilled in ice for 5 min and denatured with 0.1 mol/L NaOH by heating at 37°C for 10 min. 10 mM/L hydroquinone and 3 mol/L of sodium bisulfite were added to the denatured DNA, mixed well by hand-vortexing and incubated under mineral oil at 50°C for 14–16h. Bisulfite-modified DNA was collected on next day and kept at –20°C [21, 22].

#### *Purification of Bisulfite-modified DNA*

The bisulfite-modified DNA was collected and purified by using Wizard PCR preps DNA purification system (Promega, Madison, WI, USA) according to manufacturer's instructions with slight modifications [22].

**Table 1.** Primers and the sequences (methylated (M) and unmethylated (UM)) of p15, p16, p18 and p53 genes

Primers	Sequences
p16M	5'TTATAGAGGGTGGGGCGGATCGC 3' (sense) 5'GACCCCGAACC GCGACCGTAA 3' (antisense)
p16UM	5'TTATTAGAGGGTGGGGTGGATTGT 3' (sense) 5'CAACCCCAAACCACAACCATAA 3' (antisense)
p15M	5'GCGTTCGTATTTTGC GGTT 3' (sense) 5'CGTACAATAACCGAACGACCGA 3' (antisense)
p15UM	5'TGTGATGTGTTTGTATTTTGTGGTT 3' (sense) 5'CCATACAATAACCAAACAACCAA 3' (antisense)
p18E1 (M)	5'ATGGCCGAGCCTTGGGGGAACGAGTT 3' (sense) 5'CAACATTATTGACTTGTTCCTCCAC 3' (antisense)
p18E2 (UM)	5'AGGATTCTACCATTCTACTCTTT 3' (sense) 5'TTATTGAAGATTTGTGGCTCCCCCA 3' (antisense)
p53 E7 (M)	5'CGCGCGACTAGGGAAGACTTCTGGTCTGACTGTACCA 3' (sense) 5'AGGAAGACTTCCGAGCGGTCTGGAGTCTTCCAGTGTGA 3' (antisense)
p53 E8 (UM)	5'CGCGCGACTAGGGAAGACTTCTGGTAATCTACTGGGACGG 3' (sense) 5'AGGAAGACTTCCGAGCGGTCTCGCTTAGTGCTCCCTGG 3' (antisense)

#### Methylation-specific Polymerase Chain Reaction (MSP)

MSP was carried out based on the principle that treating DNA with sodium bisulfite would result in the conversion of unmethylated cytosine residues into uracil. Methylated cytosine residues, on the other hand, would remain unchanged. Thus, the DNA sequence of methylated and unmethylated genomic regions after bisulfite sodium conversion was distinguished by sequence-specific polymerase chain reaction (PCR) primers. The primer sequences of p15, p16, p18 and p53 genes in both methylated and unmethylated forms (table 1) have been used for PCR. 50 ng of treated DNA was amplified using specific primers for either methylated or unmethylated conditions. The thermal cycle profile was 5 min at 95 °C, 45 s at 60 °C (p15M, p15U, p16U), 65 °C (p16M), 56 °C (p18E1, p18E2) or 56 °C (p53E7, p53E8), 10 min at 72 °C and 2-min extension. 30 cycles were maintained for each PCR set-up; p18 was used as gene control.

#### Agarose Gel Electrophoresis

10 µl of each amplified product was loaded in 1.5% agarose gel, stained with 0.1% of 5 mg/ml ethidium bromide and run at 40 V and 20 mA [19]. Band intensities were measured by using 'image J' software [18].

#### Single-strand Conformation Polymorphism (SSCP) Analysis

SSCP analysis may be defined as the detection of conformational difference of single-stranded nucleotide sequences of identical length as induced by differences in the sequences under certain experimental conditions. SSCP analysis was done following the method of Bandyopadhyay et al. [23]. The denatured DNA samples were run on 12.5% polyacrylamide gel at 30 V and 15 mA for 10 h. The gel was stained with 0.5 µg/ml ethidium bromide in double-distilled water for 20 min and washed with double-distilled water for 5 min. Finally the gel was photographed under UV-transilluminator.

#### In Vivo Study

##### Experimental Animals

Healthy white Wistar rats (*Rattus norvegicus*) (below 1 month of age), weighing between 60 g and 80 g were reared in the animal house of the Department of Zoology, Kalyani University, West Bengal, India, under proper hygienic condition in polypropylene cages (temperature: 24 ± 2°C; humidity: 55 ± 5%; 12 h light/dark cycles), were allowed to drink water *ad libitum* and were set on a basal diet. The experiments were carried out as per guidelines of and clearance from the Institutional Ethical Committee, University of Kalyani (registration number 892/OC/05/CPCSEA).

#### Study Design

A randomised set of 70 rats was used for each time point and was again subdivided into 4 different groups consisting of 5 rats each as stated below:

**Group 1:** Normal: animals received normal food and water *ad libitum* (5 × 3 = 15 rats). Normal rats were sacrificed after 5th, 6th and 7th month time points.

**Group 2:** BaP-induced lung cancerous rats: animals received BaP orally, 2 days a week for 1 month and then normal diet and water (5 × 3 = 15 rats).

**Group 3:** BaP- + placebo-treated rats: animals received placebo (0.06 ml per rat) orally twice daily for the following 1, 2 or 3 months of lung cancer development (5 × 3 = 15 rats).

**Group 4:** BaP- + Condurango 30C-treated rats: animals received Condurango 30C (0.06 ml per rat) from the stock solution, orally twice daily for 1, 2 and 3 months of post-cancer intervals and were sacrificed at each fixation point (5 × 3 = 15 rats).

**Group 5:** Only placebo-treated rats: normal animals received placebo (0.06 ml per rat) orally twice daily for 1 month (5 rats).

**Group 6:** Only Condurango 30C-treated rats: normal animals received Condurango 30C (0.06 ml per rat) orally twice daily for 1 month (5 rats).

The experimental data were collected after 1 (5th), 2 (6th) and 3 (7th) months for the detection of the possible effect of Condurango 30C at post-cancer time point by sacrificing animals humanely by cervical dislocation.

#### Preparations and Administrations of BaP and Condurango 30C

BaP (dissolved in olive oil) at a dose of 50 mg/kg body weight (standardised through trial experiments) was fed to each rat through gavage [24], twice a week for 1 month; then normal diet was maintained. Lung cancer was developed after 4 months in all BaP-intoxicated rats.

1 ml of Condurango 30C from the stock solution was diluted with 20 ml of double-distilled water to make the usable diluted solution (followed by homeopathic principles). The dose was determined in relation to body weight of rats at a nearly human equivalent dose (considering average weight of human being to be around 70 kg). Each rat was fed 0.06 ml, orally from the diluted solution twice daily with the aid of a fine pipette [25] for the next 1 (5th), 2 (6th) and 3 (7th) months of lung cancer development, after 4 months as post-cancer intervals.

#### Acute Toxicity Study

Placebo- and Condurango 30C-treated rats were observed initially for 24–48 h and then for 1 month by acute toxicity study. No behavioural changes or major alterations in lung and/or liver were observed. So rats of groups 5 and 6 were denied in-depth study.

#### *Collection of Lung Samples and Isolation of Genomic DNA*

Each rat was sacrificed humanely by cervical dislocation after ending of each experimental schedule. Lung samples were collected immediately, washed with 1x PBS and stored at  $-20^{\circ}\text{C}$  for future use.

50 mg lung sample from each group (normal, cancer, placebo and drug-treated) was taken in 1.5 ml Eppendorf tubes and washed with 1x PBS. We took 50 mg lung tissue from each sample separately in a mortar, and 75  $\mu\text{l}$  of DNA extraction buffer was added to each. Then tissues were crushed with the aid of a pestle and the substance of each tissue sample was taken in separate Eppendorf tubes; 25  $\mu\text{l}$  DNA extraction buffer was again added to dissolve the substance further. Then DNA was extracted using conventional phenol/chloroform method [16]. 100  $\mu\text{l}$  1x Tris-EDTA (TE) buffer was added to DNA pellet and mixed well by vortexing.

#### *DNA Methylation Study*

DNA methylation study was done as per standard technique established by Dong et al. [21] and Ye et al. [22]. The detailed procedure of DNA methylation with methylation-specific PCR and SSCP analysis has already been explained in the in vitro study section. P15, p16 and p53 genes were used for in vivo study with p18 as gene control.

#### *Blinding*

The investigators were blinded during observation as they were observing the control or drug-treated materials in both in vitro and in vivo studies. The person who set up the experiment coded the animals/samples and did not divulge the codes unless the observer finished his observation. Then, the decoding was done before the data were analysed and compared.

#### *Statistical Analysis*

All the values in the result section represent 3 independent experiments. Values were shown as standard deviations of mean. Data were analysed and significance of the differences between the mean values was determined by one-way analysis of variance (ANOVA) with Dunnett's post-hoc tests, using SPSS 14 software (SPSS Inc, Chicago, IL, USA). Statistical significance was considered at  $p < 0.05$ . All data are presented graphically in the results and discussion section showing the significance level.

## **Results**

### *In Vitro Study*

#### *Effects of Condurango 30C on Cell Viability*

2.43/100  $\mu\text{l}$  dose of Condurango 30C was selected as  $\text{IC}_{50}$  dose for 48 h treatment against H460 cells for the entire study, previously mentioned by Sikdar et al. [19]. Cell viability of placebo-treated cells at maximum dose (5  $\mu\text{l}/100\mu\text{l}$ ) was found very close to untreated controls, also mentioned previously [19].

#### *Amplification of p15, p16, p53 and p18 Genes*

Condurango 30C-treated (2.43  $\mu\text{l}/100 \mu\text{l}$ ) DNA showed significant decrease in band intensity, specifically in methylated state, in respect to untreated and placebo-treated samples. Thus Condurango 30C showed capacity to modulate or to reduce p15 methylation in H460 cells (fig. 1A).

Results of p16 amplification showed that there was no significant alteration of p16 among untreated, placebo- and Condurango 30C-treated DNA in both unmethylated and methylated states

(fig. 1B). Condurango 30C apparently did not modulate the expression of p16 at its methylated state and thus was not considered for further study.

In case of p53 amplification, untreated DNA showed hypermethylation which was indicative of inhibition of tumour suppressor gene in cancer. On the contrary, Condurango 30C-treated DNA showed significant decrease in p53 methylation as compared to untreated and placebo-treated controls (fig. 1C).

P18 is a wild-type gene control, which was unaltered in both unmethylated and methylated conditions as compared to altered expression of other tumour suppressor genes at methylated state (fig. 1D). Results of p18 amplification showed unaltered expression in both methylated and unmethylated states in untreated, placebo and drug-treated DNA. The results shown in histograms (E, F, G, H) also support our findings.

#### *Analysis of Single-strand DNA Breakage by SSCP*

SSCP analysis refers to the denaturing of DNA into 2 single strands, but sometimes more DNA strand breakages were found due to excessive DNA damage. But in this study the amplified DNA at specific gene level was analysed by SSCP to observe the variation of DNA breakage both in number and position in drug-treated samples against untreated and placebo-treated samples, if any, at both methylated and unmethylated states of p15 and p53. In case of p15, DNA strand breakage was found in all the methylated and unmethylated states of drug-treated, placebo-treated and untreated samples. But intensity was minimal and band position was little lower in Condurango 30C-treated DNA than in other controls (fig. 1I). This qualitative data indicates [21–23] further that Condurango 30C could possibly alter p15 methylation status via epigenetic modification, i.e. without changing the DNA sequence. However, although the present findings adequately reflect the change in methylation status as a consequence of the drug response, additional data pertaining to quantitative analysis on DNA methylation would be important to confirm the extent of change in methylation status more precisely.

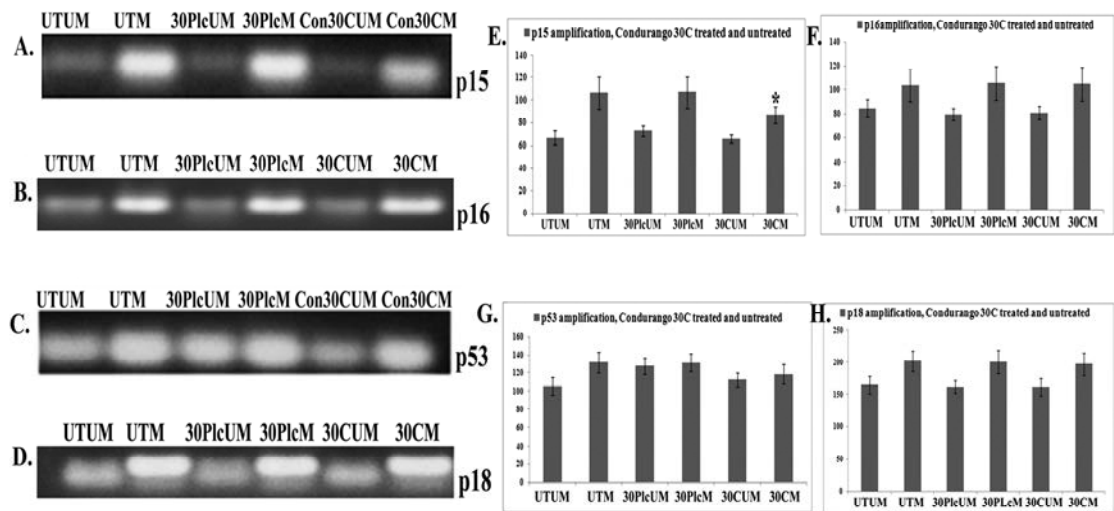
In case of p53, 2 bright single DNA strands were found in Condurango 30C-treated DNA at a little higher position of untreated and placebo-treated DNA samples, which provides further evidence for the p53 methylation modulatory function of Condurango 30C through epigenetic modification (fig. 1J).

### *In Vivo Study*

#### *Amplification of p15, p16, p53 and p18 Genes*

In the control set up, none of the rats developed cancer spontaneously until the end of 7th month. Further, no rat in the control series (untreated or treated only with placebo) showed any significant change in gene expression patterns of p15 and p53.

All rats treated with the carcinogen developed cancer. p15 amplification showed that there was no significant alteration of p15 among normal, cancer and placebo-treated DNA in both unmethylated and methylated states. But Condurango 30C-treated DNA

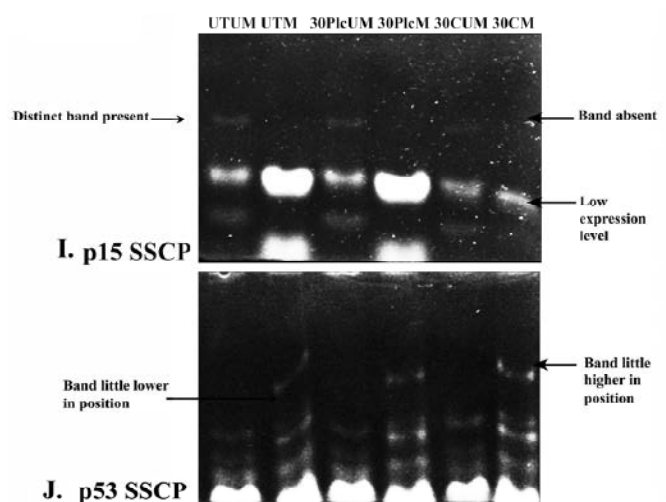


**Fig. 1.** Study on DNA methylation at specific tumour suppressor gene expression level.

**A, B)** P15 and p16 amplification show significant decrease of methylation state of p15 after Condurango 30C exposure against untreated and placebo, whereas there was no modulation of DNA methylation in p16. **C, D)** P53 and p18 amplification show significant decrease of methylation state of p53 and no such modulation in p18 against controls. The results shown in histograms **E–H** are the mean of 3 experiments  $\pm$  standard deviation.

Significance: \* $p < 0.05$  untreated (UT) versus Condurango 30C; SSCP analysis of p15 (**I**) and p53 (**J**). The figures show the DNA strand breakage in all methylated and unmethylated states of DNA in drug treated, placebo-treated and untreated samples. Yet, band intensity was minimum and band position was little lower in Condurango 30C-treated DNA (indicated by arrows) than in other controls. In case of p53, 2 bright single DNA strands were found in Condurango 30C treated DNA at a little higher position than in untreated and placebo-treated DNA samples.

UTUM = untreated unmethylated, UTM = untreated methylated, 30PlcUM = placebo unmethylated, 30PlcM = placebo methylated, 30CUM = Condurango 30C unmethylated, 30CM = Condurango 30C methylated.



showed significant decrease in band intensity, specifically in methylated condition at 5th and 6th month intervals demonstrating the ability of Condurango 30C to modulate or reduce p15 methylation in lung cancer, in vivo (fig. 2A). There was no modulation of p15 at methylated state in the placebo-treated groups at the 3 post-cancer time points of observation.

p16 amplification showed that there was no significant alteration of p16 in normal, cancer and placebo-treated DNA in both unmethylated and methylated states and in normal, cancer and Condurango 30C-treated DNA at the 3 post-cancer fixation time points. This would indicate that both the placebo and the ultra-highly diluted Condurango 30C did apparently not modulate the p16 expression at its methylated state (fig. 2B). For this reason, p16 was not considered for further study. There was no modulation of p16 methylation state in the placebo-treated groups at the 3 post-cancer fixation time points.

In Condurango 30C-treated DNA samples, expression of p53 in the methylated state was reduced especially at 5th and 6th month post-cancer time points against normal and cancerous samples, providing a positive indication of DNA methylation

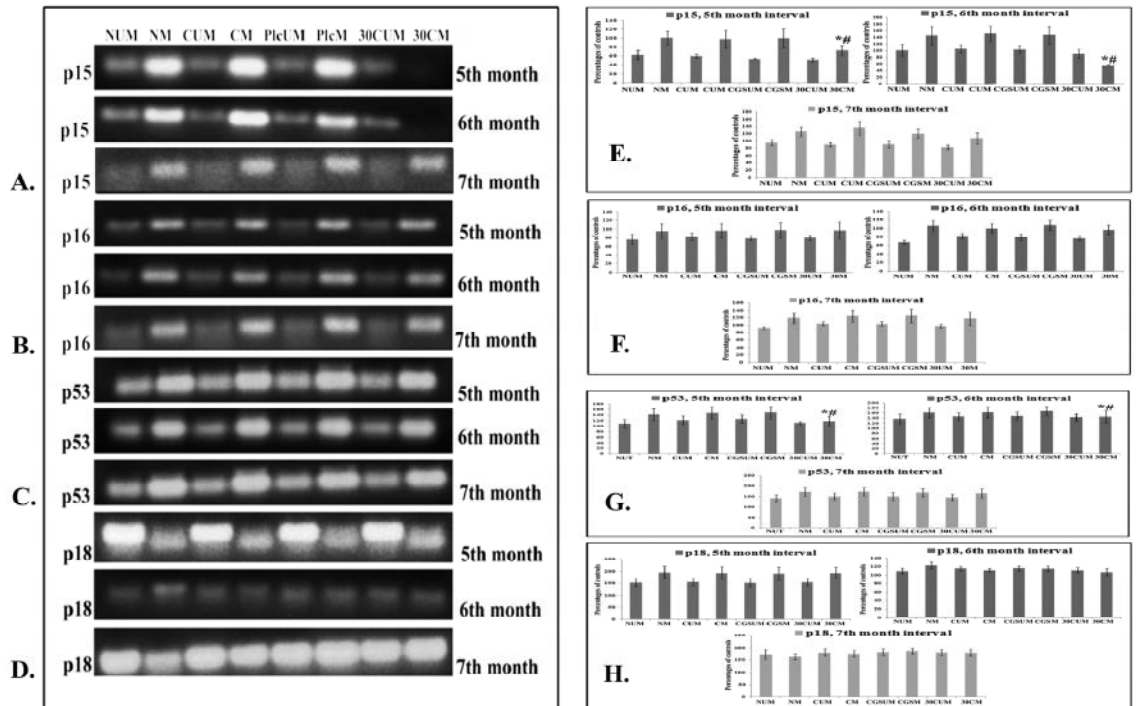
modulatory capacity of Condurango 30C in p53 gene expression (fig. 2C). There was no modulation of p53 at methylated state in the placebo-treated groups at the 3 post-cancer fixation time points.

Results of p18 amplification showed unaltered expression in both methylated and unmethylated states in placebo and Condurango 30C-treated DNA against normal and cancerous controls at the 3 different post-cancer intervals (fig. 2D). There was no modulation of p18 methylation status in the placebo-treated groups.

#### *Analysis of Single-strand DNA Breakage by SSCP*

SSCP results gave a good indication of differences in band position and single-strand breakage of p15 in case of Condurango 30C treatment at 6th month interval in regard to drug-untreated DNA from cancer-bearing rats. But in case of p53, Condurango 30C-treated DNA (6th month interval) showed one single DNA strand, the other being absent, suggesting thereby that some DNA strand modification in methylation occurred after drug treatment (fig. 3).

## Amplification of p15, p16, p53 and p18



**Fig. 2.** Study on DNA methylation at specific tumour suppressor gene expression level.

**A)** P15 amplification showed significant decrease in band intensity specifically in methylated condition at 5th and 6th month fixation time points in respect to normal and cancer-treated groups. **B)** Amplification of p16 showed that there was no significant alteration of p16 among normal and cancer-treated DNA in both unmethylated and methylated states and among normal, cancer and Condurango 30C-treated DNA in both unmethylated and methylated states at the 3 month post-cancer fixation intervals. **C)** In Condurango 30C-treated DNA samples, expression of p53 in the methylated state was reduced especially at 5th and 6th month post-cancer time points against normal and cancerous samples. **D)** Results of p18 amplification showed unaltered expression in both methylated and unmethylated states in drug treated DNA against normal and cancerous controls at 23 different post-cancer intervals. The histograms of band intensities of p15, p16, p53 and p18 are represented in **E, F, G** and **H**, respectively. The results shown in histograms are the mean of 3 experiments  $\pm$  standard deviation.

Significance: \* $p < 0.05$  normal versus Condurango 30C and # $p < 0.05$  cancer versus Condurango 30C.

NUM = normal unmethylated, NM = normal methylated, CUM = cancer unmethylated, CM = cancer methylated, PlcUM = placebo unmethylated, PlcM = placebo methylated, 30CUM = Condurango 30C unmethylated, 30CM = Condurango 30C methylated.

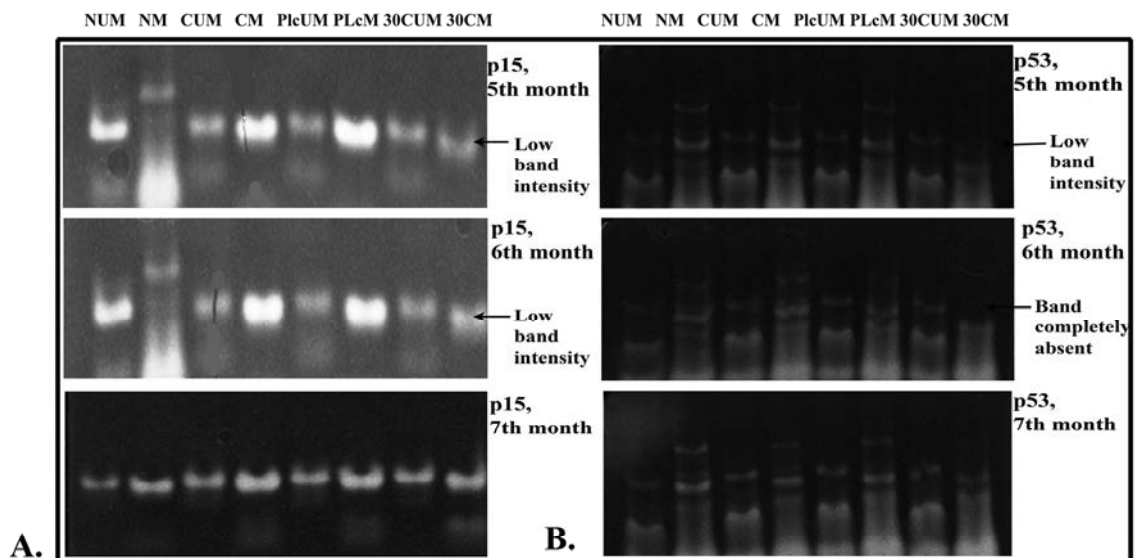
## Discussion

The results of the present study show aberrant methylation status in CpG islands of both p15 and p53 genes in both untreated and placebo-treated H460-NSCLC cells, presumably due to loss of function or inhibition of transcription of p15 and p53 genes. But Condurango 30C-treated DNA showed significant decrease in band intensity specifically in methylated condition highlighting that Condurango 30C has the capacity to modulate p15 methylation in lung cancer in vitro. In case of p53 gene, untreated and placebo-treated DNA showed hypermethylation of tumour suppressor genes, resulting in unhindered proliferation of the cancer cells. However, after Condurango 30C treatment, a significant decrease in p53 hypermethylation was noted.

DNA methylation affects all aspects of apoptosis right from its initiation to execution in different cancers [26]. Thus, DNA methylation study is important to identify the affected genes involved in apoptosis, specifically those tumour suppressor genes. We previously reported the apoptosis-inducing potential of Condurango 30C in H460 cells, in vitro and BaP-induced lung cancer of rats at

post-cancer intervals, in vivo [16, 17, 19], but it was not known if this was accomplished through change in hypermethylation status of DNA or not; this issue could now be clarified in this study.

DNA methylation is a biochemical process that is important for normal development in higher organisms. It involves the addition of a methyl group to the 5th position of the cytosine-pyrimidine ring or the number 6 nitrogen of the adenine-purine ring. Some modifications in DNA methylation status that regulate gene expression without actually changing DNA sequences are referred to as epigenetic modification. The genes with high levels of 5-mC in their promoter region are transcriptionally silent, and DNA methylation gradually accumulates long-term gene silencing. Since many tumour suppressor genes are silenced or hypermethylated by DNA methylation during carcinogenesis, there have been some attempts to repress these genes by inhibiting DNA methyltransferase enzyme [21, 22]. Aberrant methylation of CpG islands, which convert cytosine into 5-mC by the action of methyltransferase, reduces the expression of tumour suppressor genes, but does not necessarily change the gene sequence (epigenetic modification).



**Fig. 3.** SSCP analysis of **A)** p15 and **B)** p53. SSCP analysis gave a better indication of differences in band-position and single strand breakage (indicated by arrows) of p15 in case of Condurango 30C treatment at 6th month interval than drug-untreated cancerous DNA. But in case of p53-Condurango 30C-treated DNA (6th month interval) one single DNA strand was completely absent.

NUM = normal unmethylated, NM = normal methylated, CUM = cancer unmethylated, CM = cancer methylated, PlcUM = placebo unmethylated, PlcM = placebo methylated, 30CUM = Condurango 30C unmethylated, 30CM = Condurango 30C methylated.

In this study, the amplified DNA at specific gene level was analysed by SSCP to observe variations, if any, in number and position of DNA breakage in drug-treated samples as compared to that of untreated and placebo-treated samples, at both methylated and unmethylated states of p15 and p53 expression. SSCP analysis is important in rendering support to determine epigenetic modification in gene expression or cellular phenotype, caused by mechanisms other than changes in DNA sequence. In this study, SSCP analysis revealed that in case of p15, the DNA strand breaks were minimal with a low intensity; Condurango 30C-treated DNA showed p15 hypermethylation, but in case of p53, 2 bright single strands were found in Condurango 30C-treated DNA at a little higher position of the band as compared to untreated and placebo-treated DNA cells, which indicates the ability of Condurango 30C to trigger epigenetic modification in respect of expression of these 2 genes.

Thus, the present findings revealed the ability of Condurango 30C to influence methylation-associated silencing of p15 and p53 although the exact mechanism through which it was accomplished could not be properly elucidated. To get a further glimpse of whether this phenomenon was also operative in vivo condition, DNA methylation status was also examined in BaP-induced lung cancer-bearing rats, which were treated with either placebo or Condurango 30C at post-cancer intervals. P15 amplification clearly showed significantly lower expression at methylated state after post-cancer Condurango 30C treatment, specifically at 5th and 6th month time points. P53 amplification showed better modulatory activity of Condurango 30C in respect of p53 hypermethylation at 5th and 6th months of post-cancer treatment against placebo-treated samples. Thus, this result further confirmed the ability of

Condurango 30C to modulate DNA hypermethylation towards hypo- or demethylation in rats. SSCP results also supported this phenomenon by showing differences in band-position and single-strand breakage of p15 in case of Condurango 30C-treated lung samples at 6th-month interval, vis-a-vis drug-untreated cancerous and placebo-treated DNA. But in p53 Condurango 30C-treated DNA (at 6th month time point), one single DNA strand was completely absent, which possibly suggests that Condurango 30C has also DNA strand-separating abilities in methylated state and thus possible effects on epigenetic modification.

Based on our presented data and previous findings concerning Condurango's pro-apoptotic effect in vivo [16–19], Condurango's ability to potentially control cell cycle progression in BaP-induced tumours is also important here. p15 (CDKN2B) and p18 (CDKN2C) both target CDK4 and CDK6 resulting in inhibition of these kinases' ability to promote Cyclin D signalling in G1/S phase checkpoint. For the in vivo data, it appears that Condurango 30C was effective at 5th and 6th month time points in demethylating p15. This correlates well with our previous findings on apoptosis-inducing potential of Condurango [16–19]. However, it appears that this anti-tumour effect was lost at 7th month and correlates with unaltered p18 and reappearance of a p53 band at 7th month. In addition, the drop in p15 expression level in figure 3 indicates that p15 function has greatly diminished in vivo in all collected lung tissue.

Incidentally, Khuda-Bukhsh [14, 15] first proposed a hypothesis based on many direct and indirect (circumstantial) evidence that one of the mechanisms through which the homeopathic drugs act, might be caused by regulation of gene expression. The results of the present study as well as earlier evidence [27–29]



support this contention. Admittedly, more work is necessary to arrive at a definite conclusion about the precise molecular mechanism(s) of action of homeopathic drugs, but that they are endowed with the capability to trigger gene expressions appears to be more convincing now. However, more work with an open mind is warranted to learn more about the precise molecular mechanism underlying the ability of these ultra-high dilutions to effectively eradicate disease symptoms without actually carrying even a single molecule of original drug substance. An organised scientific search based on repeatable results obtained from authentic and unbiased use of methodologies from basic sciences can provide the final clarification.

## Conclusion

Modulation of hypermethylation (i.e. demethylation or hypomethylation) is a beautiful example of translational research at work. New findings on drugs capable of demethylation of some cancer-related genes can be of great help in designing new anticancer

drugs. Therefore, a search should be made to determine whether other remedies claimed of having anticancer potential also exhibit abilities of hypomethylating or demethylating certain cancer-related genes to the advantage of restricting progression or development of cancer, so that patients can live longer with fewer side effects. It seems likely that the field of epigenetic therapy will grow exponentially, and more anticancer drugs with negligible or no side effects will be discovered to give patients a better quality of life.

## Acknowledgements

This work was financially supported by a grant to Prof. A. R. Khuda-Bukhsh, Department of Zoology, University of Kalyani, by Boiron Laboratory, Lyon, France.

## Disclosure Statement

All authors declare that they have no conflict of interests.

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