Cucurbitacin-D-induced *CDK1* mRNA Up-regulation Causes Proliferation Arrest of a Non-small Cell Lung Carcinoma Cell Line (NSCLC-N6)

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Abstract. *Despite progress in chemotherapeutic agents, non-small cell lung cancers (NSCLC) still have a poor survival rate. Thus, development of new therapeutic strategies, specifically against cancer cells is still required. For this purpose, we treated the non-small cell lung cancer cell line NSCLC-N6 with the natural product cucurbitacin D (CucD) - extracted from the plant Ecballium elaterium in order first to assess its in vitro cytotoxicity, but also to study the genetic changes that it could bring out. CucD has shown a blocking in the G_1 phase of the cell cycle in NSCLC-N6 cells prior to apoptotic cell death. The reverse transcriptase-polymerase chain reaction-differential display (RT-PCR-DD) technique was also applied on treated cells to elucidate the genetic mechanisms involved. We revealed an overexpression of Cyclin-dependent kinase 1 (CDK1) mRNA after treatment and, with the use of antisense oligonucleotides, an effective role in the proliferation arrest of NSCLC-N6 cells. The present study provides new insights about the mechanisms of proliferation arrest in tumor cells and open new ways of treatment to target tumor growth.*

Progress in the treatment of non-small cell lung cancer involves the use of cisplatin in combination with paclitaxel or gemcitabin (1). Nevertheless, this cancer is still a major public health problem. Indeed, despite the progress that has been made, the remission for this type of cancer is rare; the response rate on treatment and the 5-year survival are low (2-4).

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It is also well-established, that terrestrial plants are very good sources of bioactive substances, including those active against lung cancer, such as taxanes (5, 6). In this context, the biological activity of the triterpene CucD, a natural product obtained from the aerial parts of *Ecballium elaterium* (Cucurbitaceae), has been investigated on an NSCLC-N6 cell line.

The plant *Ecballium elaterium* (L.) A. Rich. (Cucurbitaceae), also known as the 'squirting cucumber', has been a wild medicinal plant, for over 2,000 years, found abundantly in the Mediterranean region. The content of tetracyclic triterpenoids cucurbitacins (Cuc) in different parts of *E. elaterium* has been investigated by various authors (7, 8) and their pharmacological effects have been studied as well (7, 9, 10, 11) Especially, cucurbitacin D (CucD), an oxygenated tetracyclic triterpenoid found mostly in the Cucurbitaceae plant family, has also been isolated from the plant *Gonystylus keithii* (Gonystylaceae family). It has exhibited several bioactivities among which differential cytotoxicity against several cancer cell-lines (12-15).

The cell line NSCLC-N6 employed here, is derived from a human non-small-cell lung carcinoma, possesses chemosensitivity for conventional drugs close to clinical doses, making it an excellent model for studying the biological activity of new products (16). Previously, several extracts from plants or marine substances have shown biological activity on this cell line (17-23) and the properties of several chemical constituents of *Ecballium elaterium* have displayed cytostatic activity.

In the present study, the chemical constituent CucD of *Ecballium elaterium* was investigated, particularly for its cellular and molecular mechanisms of action. It has shown that it can cause arrest of the proliferation of the human NSCLC cell line. The cellular mechanisms of this inhibition have been studied extensively, especially the cell cycle blockade in G_1 phase. The genes associated with this proliferation arrest were also investigated, particularly those that could be implicated in G_1 blockade. However the

NSCLC-N6 cells have an inactive p53/p21 cascade. We have shown that this cell line has an Arg-273-His *p53* mutation and that the transactivation for the *p21* gene was inactive (24). Therefore, although these factors are often inactive in tumor cells, it is possible to direct tumor cells to the apoptotic pathway after treatment with various substances. This suggests that other genetic factors are involved in cell death. Thus, the second purpose of this study was to apply the "differential display" strategy in order to clarify the genetic mechanisms involved in the proliferation arrest of the human NSCLC-N6 cell line after treatment by CucD. This powerful strategy identifies altered gene expression at the mRNA level in any type of eukaryotic cells (25, 26). Differentially expressed genes can be selected, such as cDNA fragments obtained by PCR and then easily cloned and sequenced. Accordingly, the differential display strategy was applied using mRNA of CucD-treated cells in an attempt to identify genes implicated in proliferation arrest in G_1 phase of this human bronchopulmonary carcinoma cell line. Results showed that *CDK1* mRNA was up-regulated after treatment, and that this overexpression was involved in stopping the proliferation of the NSCLC-N6 line following CucD treatment.

Materials and Methods

Chemicals. Preparation of CucD. Cucurbitacin D (CucD) has been isolated from *Ecballium elaterium* as it has been previously reported and it has been determined through modern spectral means (Figure 1). CucD in all experiments has been dissolved as stock solution in water with 0,1% dimethyl sulphoxide (DMSO, Sigma) at 1 mg/ml before use and stored at –20˚C. For all experiments, final concentrations of the tested compound were prepared by diluting the stock with RPMI 1640 medium (Dutscher, Brumath, France). Control cultures received the carrier solvent (0.1% DMSO).

Cell line and culture. The NSCLC-N6 cell line (16) has been derived from a human non-small-cell bronchopulmonary carcinoma of a previously untreated patient (moderately differentiated, classified as T2N0M0). Cell doubling-time was approximately 48 h *in vitro* and 12 days *in vivo*. In addition, NSCLC-N6 has a mutant *P53* gene, similar to tumors *in situ*.

Cells were cultured in RPMI 1640 medium (Dutscher) supplemented with 5% fetal calf serum (Dutscher), to which were added penicillin 100 IU/ml (Dutscher), streptomycin 100 μg/ml (Dutscher) and glutamine 2 mM (Dutscher) at 37˚C in a 5% CO_2/95% air atmosphere.

Cytotoxicity determinations by continuous and discontinuous drug exposure. Experiments were performed in conditions of continuous drug exposure in 96-well microtiter plates (Falcon, Dutscher, Brumath, France). Cells (2×10^5/ml) were dropped into each well containing 50 μl of culture medium. Cell growth was estimated by a colorimetric assay based on the conversion of tetrazolium dye (MTT) (Sigma) to a blue formazan product by live mitochondria. Optical density at 570 nm, corresponding to solubilized formazan has been read for each well on a Titertek Multiskan MKII (Dutscher. Brumath. France). In a first experiment, to determine the concentration required to reduce cell growth by 50% (IC_{50}), three concentrations were tested in duplicate and cell growth was evaluated at 72 h. Four concentrations were tested in a second experiment to determine cell growth kinetics. Eight determinations of cell growth were performed for each concentration and control group at 0, 24, 48 and 72 h. For the cytotoxicity determinations by discontinuous drug exposure the experiments have been repeated. Cells were first treated during 72 h, the drug was then removed and finally MTT tests were performed at 24, 48 and 72 h post-removal.

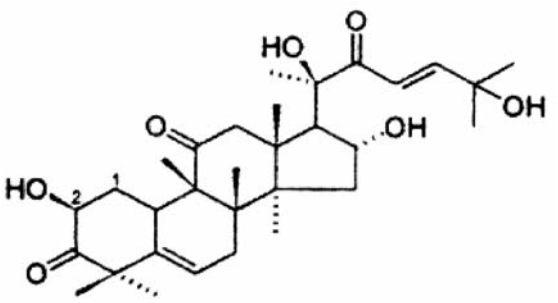


Figure 1. *Chemical structure of Cucurbitacin D.*

Analysis by flow cytometry. Briefly, NSCLC cells were plated at a concentration of 2×10^5 cells/ml in culture medium at day 0 and incubated for 24 h. At day 1, cultures were washed twice with PBS and incubated in fresh medium containing various concentrations of CucD. Treatment was continued for 48 and 72 h. DNA staining was performed using the following solution: 0.01 M glycine/NaOH, 0.96 mM propidium iodide, 0.1 M Nonidet P40, 700 IU ribonuclease A/I, 0.3 M NaCl; diluted 1:2(v/v) in PBS. The solution was dropped into plates, which were then shaken and left in the dark at 4˚C for 15 min. The cell suspension obtained after filtration was analyzed. Each control histogram has been performed with a DNA content of at least 30,000 nuclei on a Becton Dickinson FACScan (BD Bioscience. Rungis. France). To eliminate debris and DNA doublets in DNA area histogram analysis, samples were gated on the dot-plot DNA peak *versus* the DNA area, and all fluorescence histograms were established from the data included in this gate.

Differential display (DD). Cells at a concentration of 2×10^5/ml were brought to exponential growth and then treated for 72 h with CucD. Total RNA was extracted using the guanidium isothiocyanate method (27, 28). DD was performed using the RNAimage Kit (GenHunter Corp., Brookline, MA, USA), according to the manufacturer's recommendations. Briefly, total RNA pre-treated with DNAseI (Promega France, Charbonnière, France) was reverse-transcribed using three different anchor oligo-dT primers ($HT_{11}M$), followed by PCR with the same anchor oligo-dT primer and a set of eight 10-bp arbitrary primers (HAP1-8). PCR was performed in the presence of [^{33}P]-dATP (DuPont NEN, Boston MA, USA) under the following conditions: 94˚C for 5 min, 40 cycles × [94˚C, 30 s; 40˚C, 2 min; 72˚C, 30 s] followed by a 5 min extension time at 72˚C. PCR products were electrophoresed on 6% urea-containing polyacrylamide gel. Amplified fragments of treated cells and of non-treated cells RNAs were compared on adjacent lanes of the gel. The

gel was dried without fixing and exposed to X-ray film. The PCR band of interest was cut out. DNA was released from the gel by soaking it in water for 10 min and then boiling in water for 15 min. The released DNA was ethanol-precipitated and reamplified by PCR with the same primer set and one-fifth was cloned directly into PGEM-T easy vector (Promega France, Charbonnière, France). The cDNA insert was then sequenced by the dideoxynucleotide chain termination method (Sequenase 2.0, GH Healthcare Europe. Velizy-Villacoublay. France).

Riboprobe synthesis. A PCR probe (about 160 bp long) was generated from the "differential display" band of interest. Cycling conditions were the same as "differential display." The primers were the arbitrary one (5' primer) and oligo-dT, $HT_{11}M$ (3' primer), enabling amplification of the band of interest. A second amplification round was performed following gel purification of the PCR product, using a modified 3' primer: 5'GAATTCTAATACGACTCACTATAGGG AAGCTTTTTTTTTTT(M)-3'. PCR conditions with modified primer were no different than those with unmodified primer. This allowed direct use of PCR products (50-100 ng) as a template for T7 RNA polymerase (Promega France, Charbonnière, France), resulting in production of a riboprobe labeled to high specific activity with [^{33}P] ATP (DuPont NEN, Boston MA, USA). In addition to the DNA template, the 40 µl reaction mixture contained 40 mM Tris-HCl, pH 7.6, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 1 mM DTT, 40 U RNasin, 500 µM of each CTP, TTP and GTP, and 50 µCi radiolabeled alpha [^{33}P]-ATP. The reaction was initiated by adding 5-10 U of T7 RNA polymerase (Promega France), followed by incubation at 37˚C for 1 h. Ten units of DNase I (Promega France) were then added to remove the DNA template. After digestion for 15 min at 37˚C, the resulting RNA probe was purified by phenol extraction followed by ethanol precipitation. The riboprobe was then diluted in 200 µl hybridization buffer (Ambion Life technologie, Saint Aubin, France). An internal control probe, β-2 microglobulin (120 bp), was synthesized with cycling conditions as following: 94˚C for 5 min, 40 cycles [94˚C 30s, 58˚C 24s, 72˚C 30s] and primers: β2S:ACCCCCACTGAAAAAGATGA and β2AS: ATCTTCAAACCT CCATGATG. Modified reverse primer was 5-TAATACGACTCACTA TAGGGATCTTCAAACCTCCATGATG-3'.

Ribonuclease protection analysis (RPA). RPA has been performed using a commercial kit (Ambion Life technologie). Labeled RNA probe has been hybridized in solution with 10 µg of total RNA from each treated and non-treated cell as well as with 10 µg of yeast control RNA with and without RNase. Protected RNA-RNA hybrids have been extracted, precipitated and electrophoresed through a 5% denaturing polyacrylamide gel. Results' quantification has been performed, using the "Easy Win 32" (Herolab Gmbh, Wiesloc, Germany) software.

Rapid amplification of cDNA ends (RACE) PCR analysis. Total RNA (1 µg) of tumor cells treated with CucD was reverse-transcribed using 125 nM A3 specific primer P1 (5'-AAACAGTTTGATT CCCAAAGC-3') or R1 (5'TGGTTACAG TGAGACCTACACACA-3') in 20 µl of a reaction mixture (50 mM Tris-HCl pH 8.3, 40 mM KCL, 6 mM MgCl2, 1 mM dithiothreitol (DTT), 0.1 mg/ml BSA, 20 U RNasin (Promega France), 500 µM dNTPs (Promega France) containing 100 U of MMLV-reverse transcriptase (Promega France, Charbonnière, France) at 37˚C for 1 h. After a denaturing step (70˚C for 15 min), specific cDNA was purified on column (Roche, Boulogne Billancourt, France) and eluted in 100 µl distilled water. One-fifth of the reaction was polyd(C)-tailed with 10 U of terminal transferase (Promega France, Charbonnière, France) in the presence of 100 mM cacodylate buffer, pH 6.8, 1 mM $CoCl_2$, 0.1 mM DTT and 200 µM dCTP for 15 min at 37˚C, followed by a denaturation step (70˚C for 10 min). For PCR amplification, one-fifth of the d(C)-tailed cDNA was used as a template in the first reaction, employing an oligo $d(G)_{12}$ as anchoring primer and P2 (5'AATATACCTT AGAGCCTTTTTAGATGG-3') or R2 (5'CAGCTGAAGTTT GATAAC-AAAGAAA-3') specific A3 primers. PCR was conducted in a final volume of 50 µl reaction mixture containing 400 nM of each primer, 2.5 mM $MgCl_2$, 125 µM of each dNTP, and 2.5 U of Taq DNA polymerase (Promega France, Charbonnière, France) in PCR buffer (20 mM Tris, pH 8.4, 50 mM KCl). PCR was conducted under the following conditions: 94˚C for 5 min; 40 cycles x [94˚C, 1 min; 60˚C, 1 min; 72˚C, 3 min] followed by a 10-min extension time at 72˚C. The product of this reaction was diluted 1:10-1:100 and used as a template for a second nested PCR in which the same anchoring oligonucleotide and a second nested A3 specific primer were used: P3 (5 -AAATGTGT AGTTTTAAACTCAGACTCG-3) or R3 (5 -AAGACAAAAATA GACA-AGAGTTAACAA-3). Visible products in agarose gels were then cloned into PGEM-T easy vector and sequenced.

Amplification of entire sequence of CDK1 cDNA. Complementary DNA was synthesized with 1 µg total cellular RNA of treated cells and 7.6 µM of random hexanucleotide primers (Promega France) in 20 µl of a reaction mixture, as described above. PCR was conducted with 5 µl of a 1:5 dilution of cDNA in a final volume of 50 µl reaction mixture, as described above. PCR was performed using R8 (5' primer), 5'GCACTTGGCTTCAA AGCTG 3' and P3 primer (3' primer) in the following conditions: 94˚C for 5 min, 40 cycles × [94˚C, 45 s; 60˚C, 45 s; 72˚C, 1 min], followed by a 5-min extension time at 72˚C. RT-PCR was visualized through 1% agarose gel stained with ethidium bromide.

Antisense oligonucleotide studies. Antisense and sense desoxyoligonucleotides were selected according to the criteria described by Taylor (31) and Agrawal and Kandimalla (32) with Tms' ranging from 48 to 52˚C and the absence of GGGG to ensure optimal hybridization in cells cultured at 37˚C. The sequence of the anti-Cdk1 oligonucleotide (AS-Cdk1) was 5'ATAAGGACTGAGA TGATTTAAG 3' and the complementary sense oligonucleotide (S-Cdk1) was 5'CTTAAATCATCT CAGTCCTTAT 3'. The antisense oligonucleotide sequence was searched for against GenBank and no significant homologies were identified with other known sequences. Evaluation of the cell cycle distribution of N6 cells has been performed by flow cytometry, as described above, after 72 h treatment with one of each of the oligonucleotides in the absence or presence of CucD.

Statistical analysis. Data were expressed as means±standard error. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences were considered if $p \leq 0.05$.

Results

Effects of Cucurbitacin D (F5) on NSCLC-N6 cell line growth. The growth kinetics of NSCLC-N6 cells in the presence and absence of CucD at various concentrations (1 to 5 µg/ml) are shown in Figure 2. Results showed that treatment with CucD

dose-dependently inhibited NSCLC-N6 proliferation. The concentration required to reduce cell growth by 50% (IC_{50}) was 2.5±0.6 μg/ml after 72 h of treatment. The anti-proliferative effect of CucD was irreversible, since removal of the drug after 72 h of treatment did not return treated cells to their normal growth (data not shown). This latter effect suggests a progressive cell cycle blockade of N6 cells.

Effects of CucD on the cell-cycle distribution. NSCLC-N6 cells were treated with CucD for 48 and 72 h and the distribution of cells in various compartments of the cell-cycle was analyzed by flow cytometry. When treated with the test compound at 0.5 and 1 μg/ml a dose-dependent decrease in S phase was demonstrated. In addition, a third peak appears in a dose-dependent manner (8n cells) corresponding of the polyploid NSCLC-N6 cells blocked in G_1 phase, as previously described (33). Actually, histograms reached with 0.5 and 1 μg/ml show: a first peak corresponding of the 2n cells in G_1 phase, a second peak corresponding of the 4n cells blocked in G_1 and a third corresponding of 8n cells also blocked in G_1 phase. As diploid cells are more sensitive to treatment they were already entered in apoptotic cell death and the G_1 (2n) peak decreased. At 72 h and at 1 μg/ml the effect of CucD was intensified; fewer cells were in G_1 and the apoptotic cells appear on histograms just before each G_1 (2n, 4n and 8n cells) peaks (Figure 3).

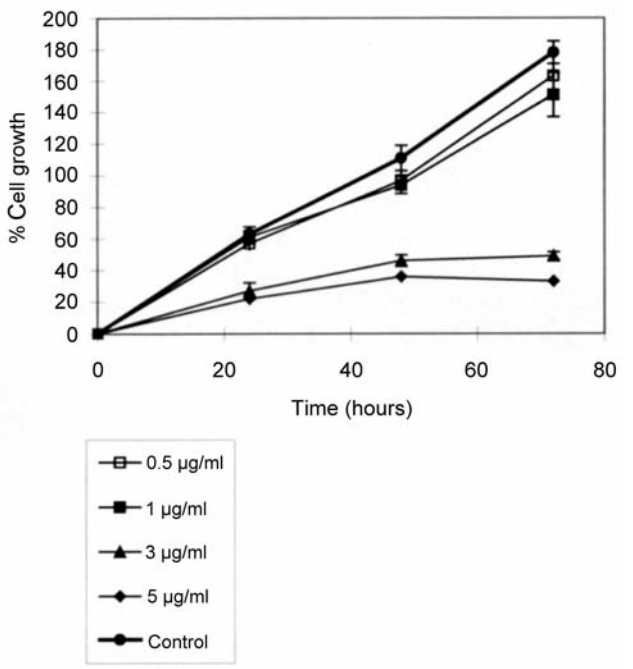


Figure 2. *Growth kinetics of NSCLC-N6 cells after CucD treatment with continuous drug exposure. Data are means±SD of three separate experiments.*

Characterization of differentially expressed genes. Among the three bands, which seemed overexpressed after treatment by CucD, only the middle band (A3) gave a positive and specific signal, by PCR reamplification with HAP3 and $HT_{11}A$ primers. The specific PCR fragment was then sequenced and initially results showed no significant homology with any sequence in GenBank/EMBL databases. This part of cDNA was then used as a probe in an RPA assay.

RPA analysis. RPA analysis of NSCLC-N6 cells with or without CucD treatment revealed that A3 expression was induced by CucD treatment (Figure 4A and 4B). These findings were in agreement with the results of "differential display" and confirmed the overexpression of A3 after arrest of NSCLC-N6 cell proliferation.

Cloning and sequencing of differentially expressed cDNA A3. Two rounds of RACE were performed to amplify the 5' region of this cDNA. The first round allowed the cloning of a fragment of approximately 900 bp (Figure 5A) and the second round yielded a 1100 bp fragment (Figure 5B). The A3 clone sequence analysis showed it was homologous in its 5'end to cdc2/*CDK1* human mRNA and was then decided to check if it was indeed the *CDK1* mRNA sequence. Thus, two primers, a 5' primer specific for cdc2/Cdk1 (R8) and a primer specific for the A3 sequence (P3) were used to generate an RT-PCR product specific for cdc2/*CDK1*. A sequence of 1900 bp corresponding to the entire mRNA of cdc2/CDK1 was amplified.

Antisense treatment. Antisense strategy was performed to investigate the implication of CDK1 in the arrest of NSCLC-N6 cell proliferation after treatment with CucD. For this experiment NSCLC-N6 cell were exposed at 3 μg/ml CucD for a highest effect in order to demonstrate a marked effect of the antisense oligonucleotide CDK1. In Figure 6, we show the results for NSCLC-N6 distribution in the cell cycle after application of 10 μM of antisense oligonucleotide. The treatment of cells with AS-Cdk1-alone (Figure 6C) modified the distribution profile of NSCLC-N6 cells after 72 h treatment compared to control cells (Figure 6A). The profile shows a greater distribution of cells in the G_1 phase of the cycle and a very marked S plateau. This is consistent with the role of CDK1 in the G_2/M transition in diploid cells (34) but shows that treatment with antisense oligonucleotide is effective on the cells. Histogram of cells exposed to CucD alone (Figure 6B) shows cells in G_1(4n) and fewer cells in G_1(2n) as shown previously in Figure 3. Cells exposed to AS-Cdk1 + CucD have a distribution profile weakly different compared to cells exposed to CucD-alone. As depicted in Figure 6D, the profile shows few cells in S and G_2/M phase whereas the 6B profile shows cells in G_1 (4n) phase only. Therefore, in the presence of antisense oligonucleotide, the

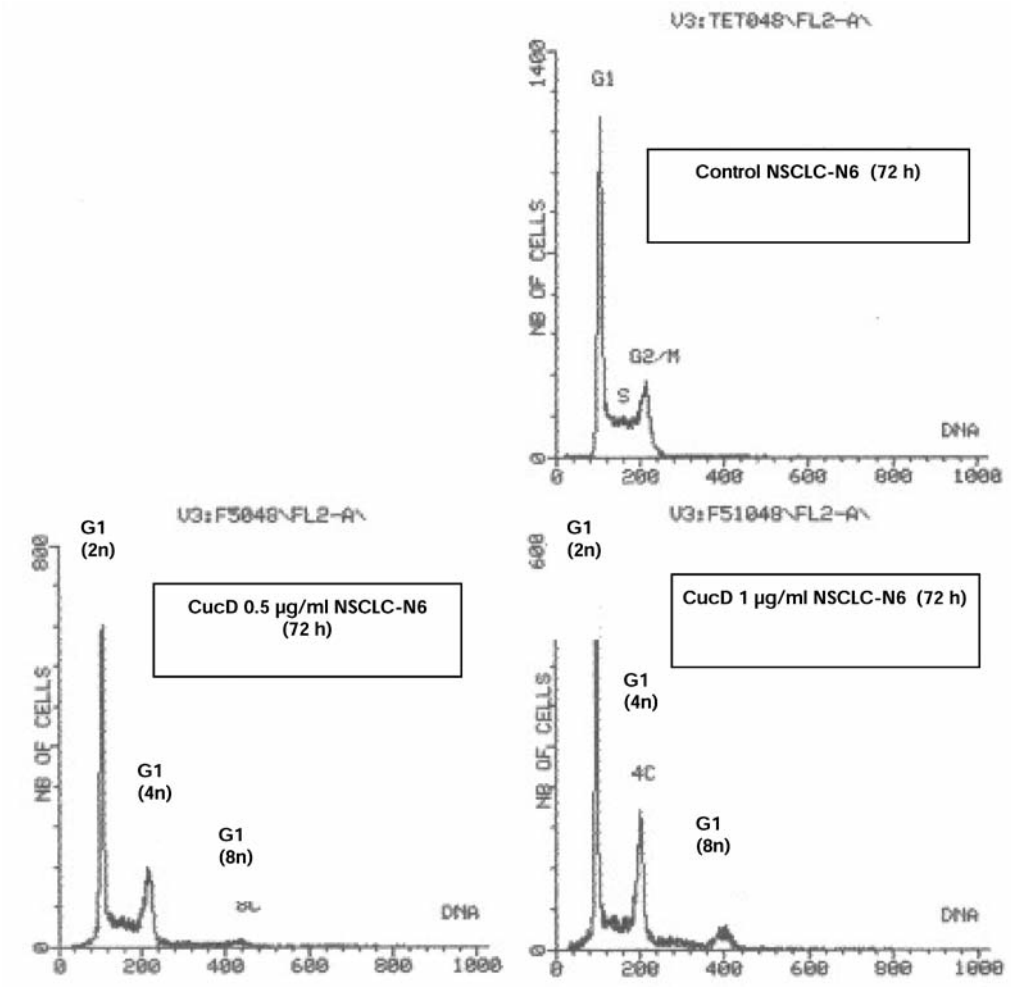


Figure 3. *Flow cytometric analysis of the DNA content of NSCLC-N6 cells cultured for 72 h in the absence (control) or presence of various concentrations of CucD. Cells were stained with propidum iodide as described in Materials and Methods.*

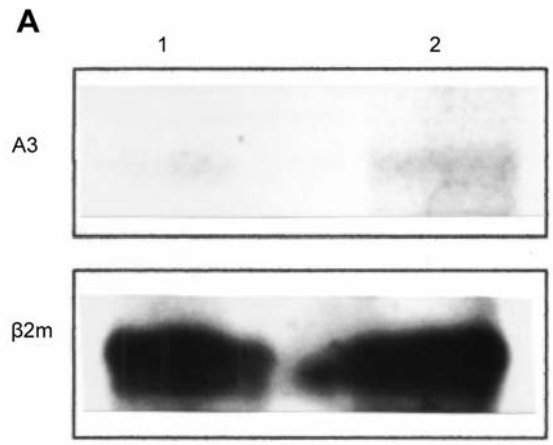
proliferation arrest and blockade in G_1 phase induced by CucD alone was decreased. Furthermore, no toxic effect was demonstrated with the control "sense" oligonucleotide (at the same dose) as it did not modify the NSCLC-N6 cell distribution profile in the cell cycle.

Discussion

In traditional medicine, cucurbitacin-containing plants have been known for their diverse pharmacological and biological activities, including their anticancer effects. These molecules have been extensively studied in the 1960s; then, this initial attention withered for the two following decades. These molecules have become the subject of renewed interest in recent years since they have been shown to inhibit STAT3, an oncogenic transcription factor, which is constitutively activated in many tumor and tumor-derived cell lines (35, 36).

The present study showed that CucD, extracted from *Ecballium elaterium*, produced irreversible cytostatic activity on the NSCLC-N6 cell line, associated with blockade in G_1 phase of the cell cycle. These findings led us to investigate the genetic mechanisms involved in this cell-cycle arrest. Our approaches were based on previous work performed in our laboratory showing the existence of genetic variations relative to known or novel genes after treatment of the NSCLC-N6 cell line by other natural cytostatic agents (37-41). As CucD shows a cytostatic activity, an attempt was made to characterize new genetic factors involved in the arrest of cell proliferation after CucD action. Thus, the "differential display" method has been applied for this purpose.

Differential expression was confirmed for the A3 fragment. As this fragment has no homology with GenBank sequences, an attempt was made to characterize the total messenger. A first RACE series gave a larger messenger



B

Probes	Untreated cells	Treated cells
A3	50	126
Beta 2m	1000	1000

Figure 4. *RPA analysis of the A3 probe in NSCLC-N6 cells. A: NSCLC-N6 cells were cultured for 3 days under the following conditions: untreated cells (1) and 2.5±0.6 μg/ml CucD (2). Total RNA was extracted and RNase protection performed using one probe for A3 and another for beta 2 microglobulin mRNA. B: Quantification of results was performed with Easy Win 32 (HRL).*

probe that did not possess an open reading frame. A second RACE series gave a larger fragment and part of its sequence corresponded to the 3' end of the open reading frame of the "cyclin-dependent kinase" Cdk1. However in the case of fragment A3, several points remain to be considered.

The sequence cloned from our experimental tumor model had a cytosine inserted in the non-translated part (in 5'). This insertion did not modify the reading frame of Cdk1 so the protein retained all its properties. However we hypothesize that this could have affected the binding of a regulatory protein.

As the expression of the CDK1 messenger was increased in CucD-treated cells in comparison to non-treated tumor cells, the antisense strategy was used to define the CDK1 function in our model and confirm its possible relation with the arrest of tumor cell proliferation. For this purpose an antisense deoxyribonucleotide sequence (oligonucleotide) was selected according to the criteria of Taylor (31) and Agrawal *et al*. (32). The distribution of cells in the cycle has been modified by the activity of the antisense oligonucleotide compared with control. This proves the effectiveness of the technique. Furthermore, in the presence of the combination antisense and CucD, the distribution profile of cells weakly changed, compared to those treated with CucD-alone. The blockade induced by CucD alone in G_1 phase was of a lower magnitude as indicated by flow cytometry analysis, observed as a reduction in the G_1 peak of polyploidic cells (4n chromosomes). Oligonucleotide activity played a "recruiting" role for cells in the cycle and was competitive with CucD activity. The low activity of the antisense treatment recorded in this study may be explained by the compensation of the two opposite effects between CucD and oligonucleotides. In conjunction with this study,

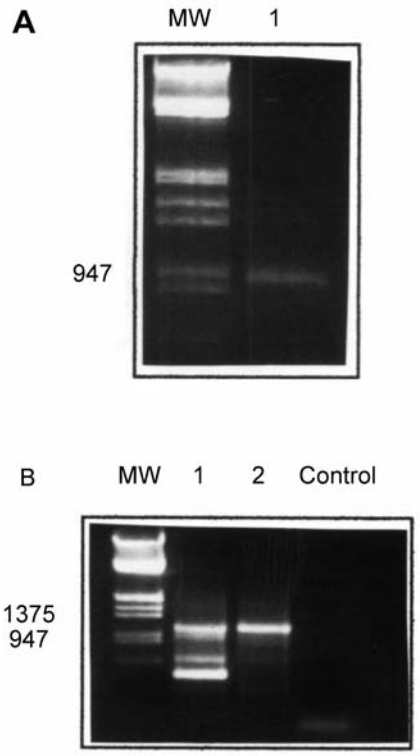


Figure 5. *Results of RACE PCR electrophoresed in 2% agarose gel and stained with ethidium bromide. A: First round of RACE PCR. RACE PCR products were generated, as described in the Materials and Methods with P3 and oligo d(G)12 primer by a nested PCR on the P2-oligo d(G)12 amplification (lane 1). B: Second round of RACE PCR: first PCR with R2 and oligod(G) primer has generated non-specific bands (lane 1). Nested PCR (with R3 - oligo d(G)12 primers) generated a specific band of 1100pb (lane2) on this sample. MW lane shows the lambda phage DNA Eco RI-Hind III digest as molecular weight marker. The control lane shows amplification without the DNA sample.*

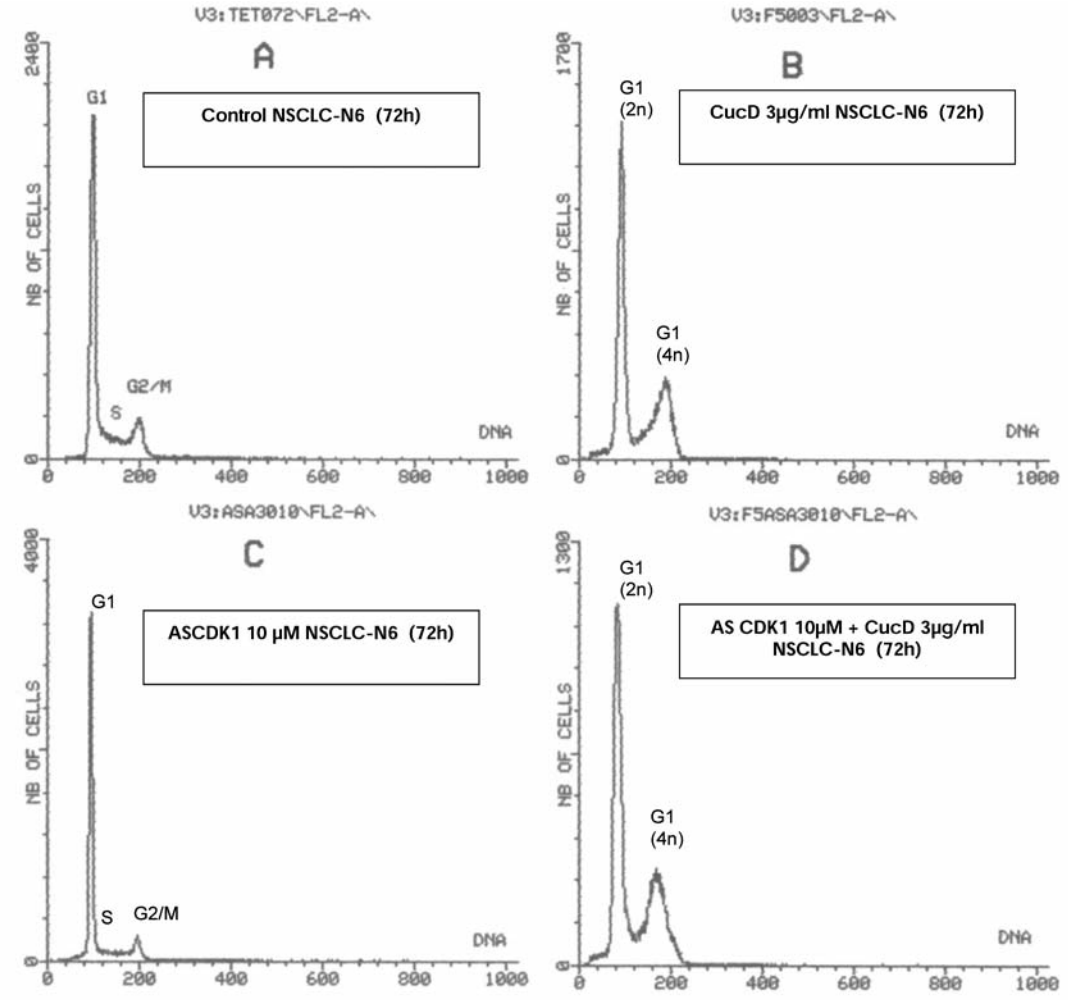


Figure 6. *Flow cytometric analysis of the DNA content of NSCLC-N6 cells cultured for 48 h in the absence (control) (A) or presence of CucD (3 µg/ml) and /or Cdk1 antisense oligonucleotide (10 µM) (C and D). Cells were stained with propidium iodide as described in Materials and Methods.*

the choice of a control sequence (sense sequence) allowed us to validate our investigation concerning functional approach of CDK1 in our model. Thus, in terms of antisense strategy, results showed that CDK1 plays a role in the arrest of NSCLC-N6 cell proliferation after induction subsequent to treatment.

Human *CDK1*, has been originally cloned by Lee and Nurse (42). The gene codes for a serine/threonine kinase, which in mammals is involved in control of G_2-M transition in the cell cycle and mitosis. Its kinase activity is activated in association with cyclin A during G_2-M transition and with cyclin B during mitosis. The CDK1-cycline complex acts as a maturation promoting factor (MPF), as described in yeast, performing a veritable mitosis-initiating element responsible for the phosphorylation of various substrates (43-45). Only CDK1 newly-synthesized during G_1/S transition can show kinase activity during mitosis. Activation of the transcription of the *CDK1* gene has been detected at each step of G_1/S transition in the cell cycle, and this new- synthesized CDK1 is then active during mitosis (46).

In yeast, *Cdk1* ($p34^{cdc2}$ for *Saccharomyces pombe* and $p34^{cdc28}$ for *Saccharomyces cerevisiae*) has a central role in progression of the different phases of the cell cycle. Fifteen years ago, it was proposed that the cell cycle in yeast can be driven by quantitative changes in the activity of a single protein kinase complex comprising a cyclin and cyclin-dependent kinase 1. *CDK1* expression that starts in late G_1 through S phase is triggered by an intermediate level of CDK1 activity, while mitosis depends on a higher kinase activity (47, 48). The following cycle can then occur only if CDK1 activity drops again under the minimal threshold level.

Recently, the central role of the CDK1 activity in eukaryotic cells has emerged. For example in hepatocytes the concomitant expression and activation of both CDK1 and CDK2 were shown at the G_1/S transition and these two CDKs contribute to DNA replication (44). *In vivo*, after a decade of extensive work on gene-knockout mouse models of cell-cycle regulators, several unexpected compensatory mechanisms were uncovered among cyclins and CDKs, but of the twenty CDKs that have –so far– been identified in humans, CDK1 is the only one that is essential for the cell cycle in all eukaryotic cells. Similar to yeast, it was recently discovered that CDK1-alone can drive the mammalian cell cycle in a quantitative model (34, 49).

In the light of data in literature, it is interesting to consider the possible mechanisms linking the arrest of proliferation of the cell cycle after treatment of NSCLC-N6 cells by CucD with subsequent induction of CDK1. In fact, the arrest of cells in G_1 phase after CucD action may be due to an aberrant (premature) accumulation of CDK1 messenger during G_1 phase and a CDK1 activity over the intermediate level required for G_1/S transition. Furthermore this is consistent with the fact that *p53* is mutated and inactive in the NSCLC-N6 cell line (24) as *p53* is known to negatively regulate CDK1 transcription (50).

Currently more studies demonstrate the pro-apoptotic role of CDK1. The first study conducted by Shi *et al*. (51) showed that premature activation of Cdk1 kinase was required during apoptosis induced by a lymphocyte granule protease (fragmentin-2) in an *in vitro* lymphoma model. Today several studies report the CDK1's activating role in the paclitaxel-induced apoptotic cell death. It participates also in HIV-1-induced apoptosis and an unscheduled CDK1 activation may contribute to neuronal apoptosis occurring in neurodegenerative diseases (52, 53). Thus, we suggest that the inappropriate expression of CDK1 in NSCLC-N6 cells in the G_1 phase and its highly expressed activity promote the entry of the cells in the apoptosis pathway.

In summary, the present study suggests that CucD is a growth inhibitor of NSCLC-N6 lung cancer cells *in vitro* by inducing the overexpression of *CDK1* gene that promotes cell cycle arrest at the G_1 phase and subsequent induction of apoptosis.

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