Int. J. Cancer: **92,** 388–397 (2001)

Publication of the International Union Against Cancer

UP-REGULATION OF A NOVEL mRNA (NY-CO-1) INVOLVED IN THE METHYL 4-METHOXY-3-(3-METHYL-2-BUTENOYL) BENZOATE (VT1)-INDUCED PROLIFERATION ARREST OF A NON-SMALL-CELL LUNG CARCINOMA CELL LINE (NSCLC-N6)

D. CARBONNELLE[1], C. JACQUOT[1], X. LANCO[1], G. LE DEZ[1], C. TOMASONI[1], G. BRIAND[1], A. TSOTINIS[2], T. CALOGEROPOULOU[3] and C. ROUSSAKIS[1]*

[1]*ISOMer (Institut des substances et organismes de la mer), Laboratoire de Pharmacologie Marine, Faculté de Pharmacie, Nantes, France*
[2]*Laboratory of Pharmaceutical Chemistry, School of Pharmacy, University of Athens, Athens, Greece*
[3]*National Hellenic Research Foundation (NI-IRF) 48 Vas, Constantinou Avenue, 116-35 Athens, Greece*

It is now well known that treatment of tumors, especially non-small-cell lung cancer (NSCLC), remains limited and it is urgent to develop strategies that target tumor cells and their genetic features. In this regard, our work is about genetic modifications arising in an *in vitro* NSCLC cell line after treatment with a chemical substance, methyl 4-methoxy-3-(3-methyl-2-butenoyl) benzoate (VT1). First, we showed that VT1 induces arrest of proliferation by blocking cells in the G1 phase of the cell cycle. Second, we use "differential display" strategy to clarify the genetic mechanisms involved in this proliferation arrest. A novel mRNA, NY-CO-1 (New-York Colon 1), of unknown function showed up-regulated expression after treatment. Application of "antisense" strategy confirmed this novel mRNA induction was effectively linked to growth arrest. Therefore, these data provide new information about mechanisms participating in arrest of proliferation of tumor cells and open new ways of treatment to target tumor growth.

Key words: *bronchopulmonary carcinoma; NSCLC-N6 gene expression; VT1; NY-CO-1; differential display*

The use of chemotherapeutic agents in the treatment of tumors is limited by several factors including innate or acquired drug resistance and systemic toxicity.[1] In bronchopulmonary carcinomas, chemotherapy remains relatively inactive against these cancers, with a poor response rate and a survival benefit limited to a period of a few weeks or months.[2,3] Accordingly, new treatment approaches need to be considered.

In this context, the first purpose of our study was to investigate the biological activity of a chemical constituent, methyl 4-methoxy-3-(3-methyl-2-butenoyl) benzoate, also named VT1, in an NSCLC cell line derived from a human non-small-cell lung carcinoma. VT1 is a synthetic molecule obtained after chemical modifications of a precursor molecule of natural origin extracted from *Piper-Toboganum*.[4] The NSCLC cell line shows a chemosensitivity for conventional drugs close to clinical doses, making it an excellent model for studying the biological activity of new products,[5] including marine substances that have shown biological activity on this cell line.[6–8]

The present study, which investigated VT1 cellular and molecular mechanisms of action on the human NSCLC cell line, found that proliferation could be stopped by incubation with VT1. The cellular mechanisms involved induction of programmed cell death of apoptotic type, which was associated with cellular morphological modifications and cell cycle blockade in the G1 phase.

Many studies have presented previously genetic factors involved in cell death. They include nuclear factors like p53 and its partners, p21 and GADD45,[9] but also cytoplasmic and extracellular factors like the bcl-2 gene family,[10] caspases[11] or FAS ligand and TNFα.[12] In tumor cells these factors are often inactive (mutation in the p53 gene), but it is possible to engage these cells in a programmed cell death after treatment by a certain type of substance. The latter shows that other genetic factors implicated in programmed cell death should exist and, finally, that tumor cells are a good model for characterizing them.

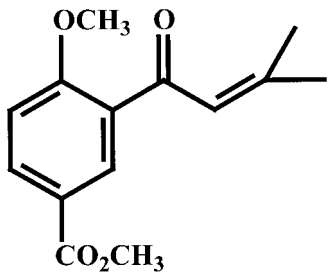


FIGURE 1 – Chemical structure of VT1, methyl -4-methoxy-3-(3-methyl-2-butenoyl) benzoate.

Thus, the second purpose of our study was to clarify the genetic mechanisms involved in proliferation arrest of the human NSCLC-N6 cell line after treatment, which led us to employ the "differential display" strategy. This powerful method makes it possible to identify altered gene expression at the mRNA level in any eukaryotic cells.[13] Differentially expressed genes can be selected, such as cDNA fragment obtained by PCR, and then easily cloned and sequenced. Accordingly, the differential display method using mRNA of VT1-treated cells was performed to clone differentially expressed genes, as compared with untreated cells, and to identify the genes implicated in proliferation arrest in the G1 phase of this human bronchopulmonary carcinoma cell line.

MATERIAL AND METHODS

Chemical substances

Methyl 4-methoxy-3-(3-methyl-2-butenoyl) benzoate (Patent PCT: w099/54278, 28 october 1999), also named VT1, was dissolved in EPPI at 1 mg/mL before use. Its chemical structure is presented in Figure 1.

*Correspondence to: ISOMer (Institut des substances et organismes de la mer), Laboratoire de Pharmacologie Marine, Faculté de Pharmacie, BP 53508, 44035 Nantes cedex 1, France. Fax: +33-2511-25690.
E-mail: christos.roussakis@sante.univ-nantes.fr

Received 11 July 2000; Revised 12 September 2000; Accepted 16 October 1999

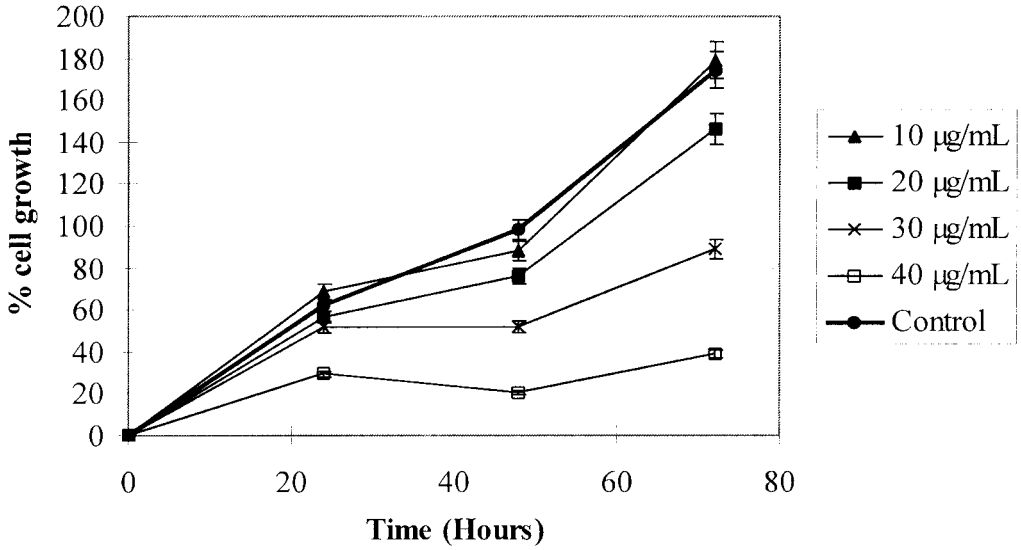


FIGURE 2 – Growth kinetics of NSCLC-N6 cells after VT1 treatment with continuous drug exposure.

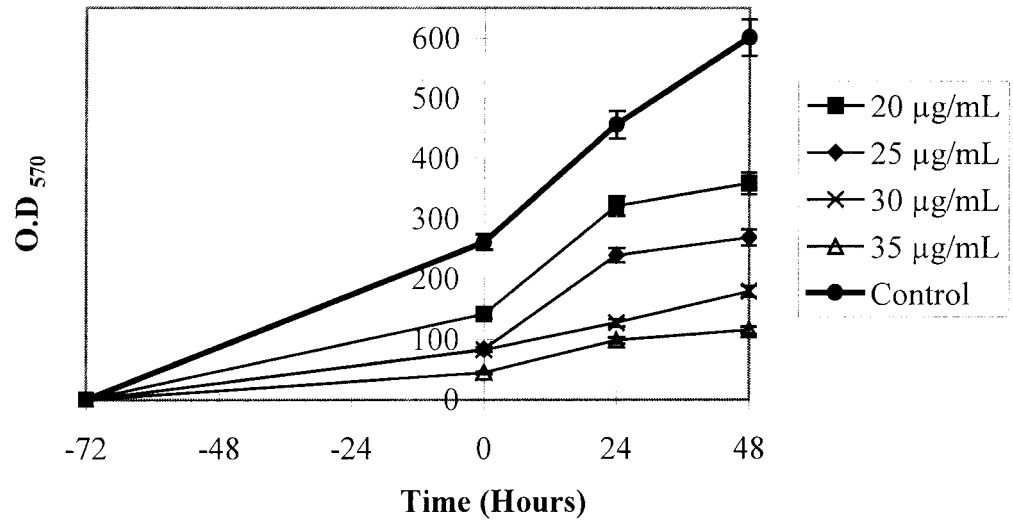


FIGURE 3 – Growth kinetics of NSCLC-N6 cells after VT1 treatment with discontinuous drug exposure.

Cell line and culture

The NSCLC-N6 cell line[5] was derived from a human non-small-cell bronchopulmonary carcinoma of a previously untreated patient (moderately differentiated, occasionally classified as T2N0M0). Cell doubling time was approx. 48 hr *in vitro* and 12 days *in vivo*. Cells were cultured in RPMI 1640 medium (Seromed, Poly Labo, Strasbourg, France) supplemented with 5% FCS, to which were added penicillin 100 IU /mL, streptomycin 100 µg/mL and glutamine 2mM at 37°C in 5% CO_2/95% air atmosphere.

Cytotoxicity determinations by continuous drug exposure

Experiments were performed in conditions of continuous drug exposure in 96-well microtiter plates (Falcon, Poly Labo). In each well, 2×10^5 cells/mL were placed containing 50 µL of culture medium. Cell growth was estimated by a colorimetric assay based on the conversion of tetrazolium dye (MTT) (Sigma, Saint Quentin Fallavier, France) to a blue formazan product by live mitochondria.[14] Optical density at 570 nM corresponding to solubilized formazan was read for each well on a Titertek Multiskan MKII.

A first experiment was done to determine the IC 50. Three concentrations were tested in duplicate and cell growth was evaluated at 72 hr. A second experiment was done to give a kinetic of cellular growth. Four concentrations were tested. Eight determinations of cellular growth were performed for each concentration and control group at 0, 24, 48 and 72 hr. Percentage cell growth was estimated according to the formula :

$$100 \times (O.D_{570}\ \text{experimental (xh)} - O.D_{570}\ \text{control (0 hr)} \div O.D_{570}\ \text{control (0 hr)})$$

Cytotoxicity determinations by discontinuous drug exposure

Cells were incubated for 72 hr (2×10^5 cells/mL) in microtiter plates in the culture condition described above and in the presence or absence of drug. After removal of medium, cells were washed twice with phosphate-buffered saline (PBS) to eliminate drug traces. Then 100 µL of fresh culture medium containing no drug was added to cells. Cell growth was evaluated, as described above, on this day (considered as day 0) and at 24, 48 and 72 hr.

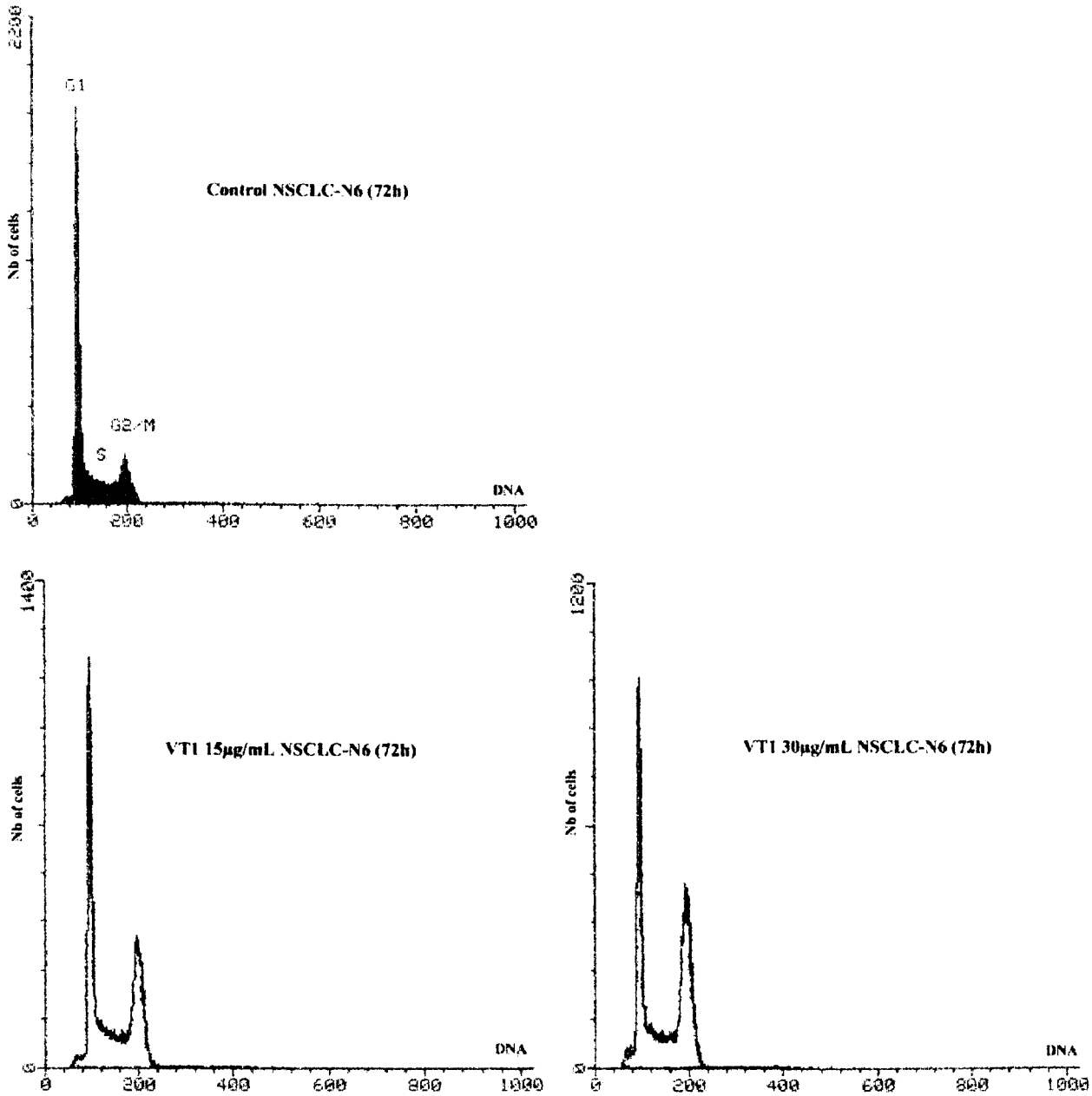


FIGURE 4 – Histograms of the DNA of NSCLC-N6 cells cultured for 72 hr in the absence (control) and presence of different VT1 concentrations.

Flow cytometry assay

Cells were cultured in 6-well plates (Falcon, Poly Labo) at a concentration of 2×10^5 cells/mL in culture medium at day 0. At day 1, cultures were washed twice with PBS and incubated in fresh medium containing 15 µg/mL to 20 µg/mL VT1. Treatment was continued for 72 hr. DNA staining was carried out using the technique of Vindelov.[15] The solution (glycine/NaOH 0.01 M; propidium iodide 9.6×10^{-5} M; nonidet P40 0.1 M; ribonuclease A/I 700 IU; NaCl 0.3 M; diluted 1:2(v/v) in PBS) was dropped into plates, which were then shaken and left in the dark at 4°C for 15 min. The cell suspension obtained was filtered on nylon mesh (50 µm) and analyzed. Each control histogram was performed with the DNA content of at least 30,000 nuclei on a Becton Dickinson (France) FACscan. To eliminate cellular debris and DNA doublets on DNA area histogram analysis, samples were gated on the dot-plot DNA peak vs. DNA area, and all fluorescence histograms were established from the data included in this gate.

Morphological analysis

Cells at a concentration of 2×10^5/mL were brought to exponential growth and then treated for 72 hr with VT1 and examined and photographed in a microscope (Olympus, OSI, France).

RNA samples

Total cell line RNA. Cells at a concentration of 2×10^5 cells/mL were brought to exponential growth and then treated for 72 hr with VT1. Total RNA was extracted using the guanidium isothiocyanate method.[16]

Tumor samples RNA. Xenografts of female nude NMRI/nu/nu mice with N6 cells were performed as described elsewhere.[17]

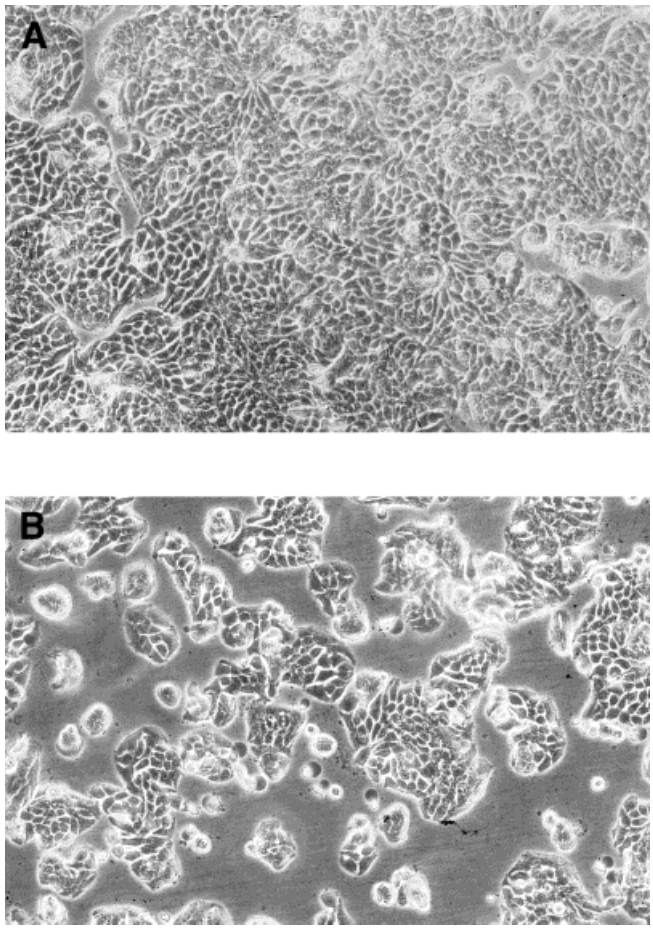


FIGURE 5 – Effects of VT1 treatment on NSCLC-N6 cells. (*a*) General view of untreated tumor-cell culture (×100), which shows interdependent cells. (*b*) General view of tumor-cell culture treated with VT1 (7 μg/mL) during 72 hr (×100) with appearance of cytoplasmic cavities.

Groups of 6 mice were selected for each concentration. After 3 weeks of treatment, fresh tumor biopsies were obtained following dissection. Total RNA was extracted by guanidium isothiocyanate method from tumor biopsies of the best responsive mice in each group.

Differential display (DD)

DD was performed using the RNAimage Kit (GenHunter, Brookline, MA) according to the manufacturer's recommendations. Briefly, total RNA pretreated with DNAseI (Appligene, France) was reverse-transcribed using 3 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 under the following conditions: 94°C for 5 min., 40 cycles × [94°C, 30 sec; 40°C, 2 min; 72°C, 30 sec] followed by 5 min extension time at 72°C. The PCR products were electrophoresed on 6% urea-containing polyacrylamide gel. Amplified fragments of treated cells' RNAs and nontreated 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 from the gel. 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 cloned directly into PGEM-T easy vector (Promega, Charbonnieres, France). The cDNA insert was then sequenced using the dideoxynucleotide chain termination method (Sequenase 2.0, Amersham, Saclay, France).

Riboprobe synthesis

A PCR probe was generated from the "differential display" band of interest. Cycling conditions were the same as "differential display," The primers were the arbitrary primer (5′ primer) and oligo-dT, $HT_{11}M$ (3′ primer) that enabled amplification of the band of interest. A second round of amplification was performed following gel purification of the PCR product using a modified 3′ primer: 5′GAATTCTAATACGACTCACTATAGGGAAGCTTTTTTTTTTT(M)-3′. PCR conditions with the modified primer were no different from the unmodified primer. This allowed direct use of PCR products (50–100 ng) as template for the T7 RNA polymerase (Promega), resulting in production of a riboprobe labeled to high specific activity with [^{33}P] ATP (Du Pont NEN, France). 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 of RNasin and 500 μM each of CTP, TTP and GTP, and 50 μCi radiolabeled alpha ^{33}P-ATP was added. Reaction was initiated by adding 5–10 U of T7 RNA polymerase (Promega) followed by incubation at 37&δεγ;C. for 1 hr. Ten units of DNase I (Promega) were then added to remove 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, Inc., Clinisciences, France). An internal control probe, β-2 microglobulin (120bp), was synthesized with cycling conditions and primer as described (Bösch et al., 1998). Modified reverse primer was : 5-TAATACGACTCACTATAGGGATCTTCAAACCTCCATGATG-3'.

Ribonuclease Protection Analysis (RPA)

RPA was performed using a commercial kit (Ambion). A labeled probe was hybridized in solution with 10 μg of total RNA from each treated and nontreated cells as well as with 10 μg of yeast control RNA with and without RNAse. Protected RNA-RNA hybrids were extracted, precipitated and electrophoresed through a 5% denaturing polyacrylamide gel. Quantification of the results was performed using the "Easy Win 32" (Herolab, Fisher Scientific, Elancourt, France.) software.

Antisense oligonucleotide studies

Antisense oligonucleotides were selected according to the criteria described by Thompson *et al.*[19] with Tm's ranging from 48°–52°C to ensure optimal hybridization in cells cultured at 37°C. The sequence of the antisense oligonucleotide (AS-NY-CO-1) was 5′ TGGTCAACCTGAGTCAT 3′ and complementary to the translation initiation site. The complementary sense oligonucleotide (S-NY-CO-1) was 5′ ATGACTCAGGTTGACCA 3′. The antisense oligonucleotide sequence was searched against GenBank, and no significant homologies were identified with other known sequences. Two types of analysis were performed: First, cellular growth kinetic of N6 cells was carried out, as described above, using various concentrations of antisense and sense oligonucleotides. Second, evaluation of cell-cycle distribution of N6 cells was performed using flow cytometry, as described before, after 72 hr treatment with one of each oligonucleotides in the absence or presence of VT1.

RESULTS

Effects of VT1 on the NSCLC-N6 cell line

Antiproliferative effect of VT1. The concentration required to reduce cell growth by 50% (IC50) was determined as described above and was 23 μg/mL after 72 hr of treatment. The growth kinetics of NSCLC-N6 cells in the presence and absence of VT1 at various concentrations (10 to 40 μg/mL) are shown in Figure 2. Nontreated cells showed a classical kinetic pattern: a growth phase during 24 hr followed by a plateau phase. Conversely, cells treated with VT1 did

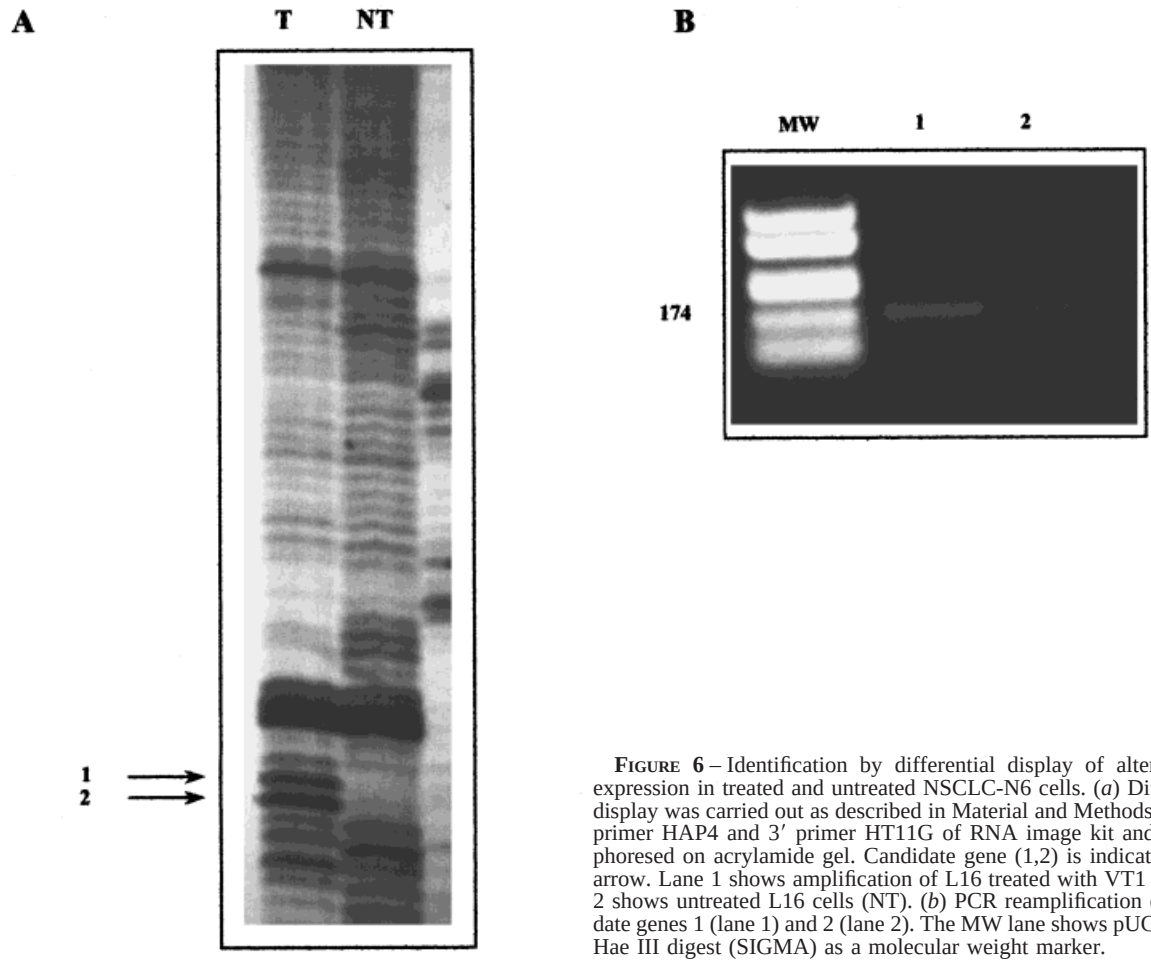
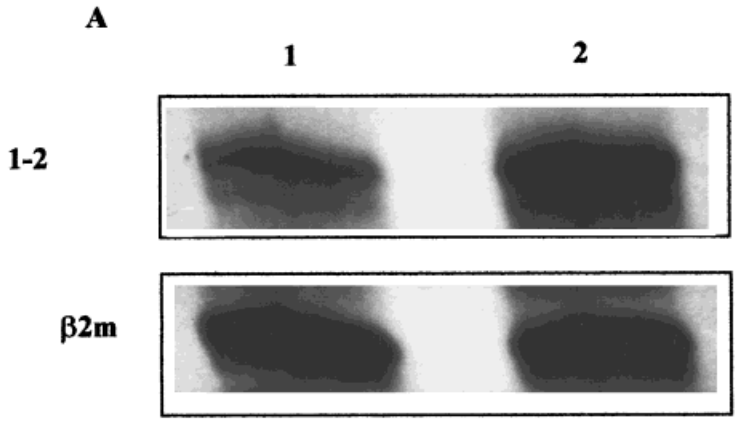


FIGURE 6 – Identification by differential display of altered gene expression in treated and untreated NSCLC-N6 cells. (*a*) Differential display was carried out as described in Material and Methods using 5′ primer HAP4 and 3′ primer HT11G of RNA image kit and electrophoresed on acrylamide gel. Candidate gene (1,2) is indicated by an arrow. Lane 1 shows amplification of L16 treated with VT1 (T); lane 2 shows untreated L16 cells (NT). (*b*) PCR reamplification of candidate genes 1 (lane 1) and 2 (lane 2). The MW lane shows pUC18 DNA Hae III digest (SIGMA) as a molecular weight marker.



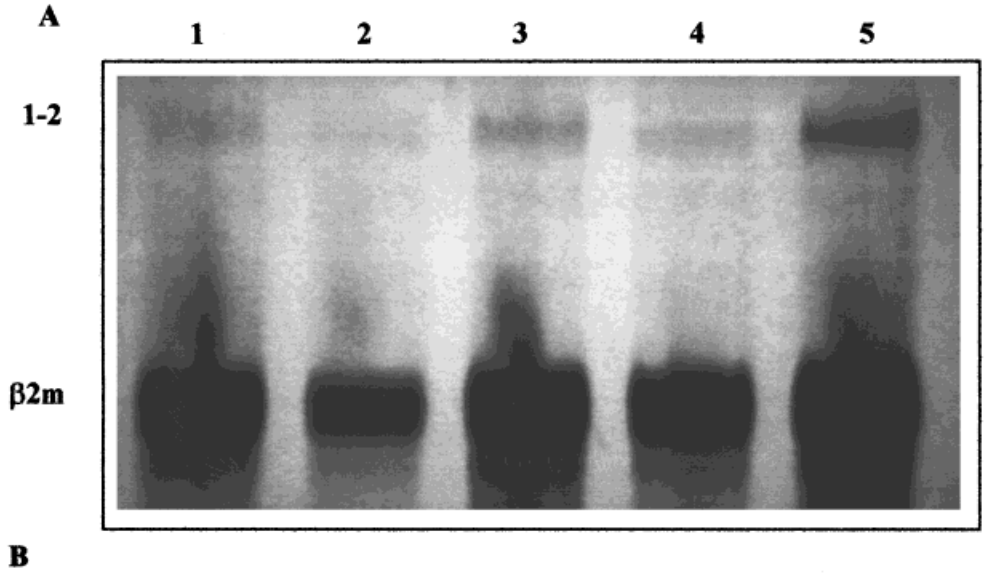
B

Probes	(1) Untreated cells	(2) Treated cells
1-2	510	1450
Beta 2m	1000	1000

FIGURE 7 – RPA analysis of 1–2 probe in NSCLC-N6 cells. (*a*) NSCLC-N6 cells were cultured for 3 days under the following conditions: untreated cells (1) and 7 μg/mL VT1 (2). Total RNA was extracted and Rnase protection performed using 1 probe for the 1–2 fragment and another for the Beta 2 microglobulin mRNA. (*b*) Quantification of results with Easy Win 32 (HRL) software.

not proliferate and the inhibitory effects of VT1 on N6 growth were dose-dependent. Figure 3 shows that this antiproliferative effect was reversible for lower doses (20 μg/mL, 25 μg/mL) and irreversible for highest doses (removal of drug after 72 hr of treatment did not induce treated cells to recover normal growth). This latter effect suggests a progressive cell cycle blockade of N6 cells.

Effects of VT1 on the NSCLC-N6 cell cycle. The effects of VT1 on the cell cycle after 72 hr of treatment are shown in Figure 4. A blockade of cells in the G1 phase was demonstrated by a dose-dependent decrease in the S phase. At lower doses, partial blockade occurred in the G1 phase together with the appearance of polyploidy (as indicated by an 8C peak). At 30 μg/mL, 8C cells were nonexistent, though present at intermediary doses. For the highest dose, S phase breakdown was observed with a reduction in the G2 phase typical of a G1 blockade of diploid cells.



Probes	1 (Control)	2 (Control)	3 (IV 60mg/kg VT1)	4 (IP 100mg/kg VT1)	5 (IP 150mg/kg VT1)
1-2	62	51	112	77	176
Beta 2m	1000	1000	1000	1000	1000

FIGURE 8 – RPA analysis of expression of the 1–2 probe in tumor samples. (*a*) NSCLC-N6 cells were xenografted in nude mice as described in Material and Methods and mice were treated under the following conditions: control untreated mice cells (lanes 1,2), mice intravenously treated with 60 mg/kg VT1 (lane 3) and mice intraperitoneally treated with 100 mg/kg (lane 4) and 150 mg/kg (lane 5) VT1. Total RNA was extracted from tumor samples and Rnase protection performed using 1 probe for the 1–2 fragment and another for the Beta 2 microglobulin mRNA. (*b*) Quantification of results with Easy Win 32 (HRL) software.

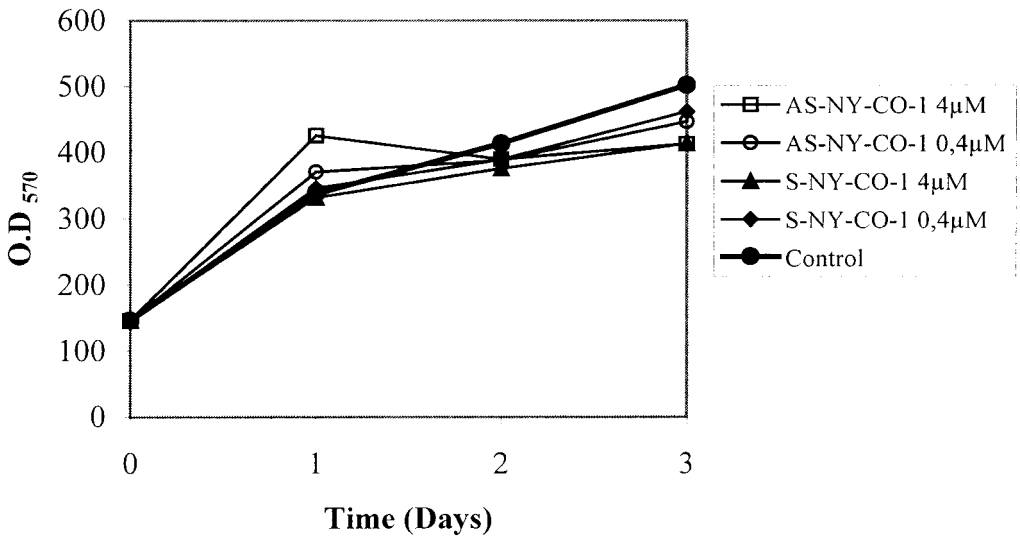


FIGURE 9 – Growth kinetics of NSCLC-N6 cells in the absence (control) and in the presence of different concentrations of antisense and sense NY-CO-1 oligonucleotides.

Effects of VT1 on the NSCLC-N6 cell line morphology. Figure 5 shows microscopic observations of the NSCLC-N6 N6 cells cultured for 72 hr in the absence and presence of VT1 (7 μg/mL). Without treatment, cells form an interdependent group and isolated cells occur infrequently (Fig. 5*a*). In presence of VT1, cells show various modifications of the cytoskeleton: increase of the intracytoplasmic volume, reduction of nuclear diameter and volume and apparition of important intracytoplasmic vacuoles (Fig. 5*b*). These

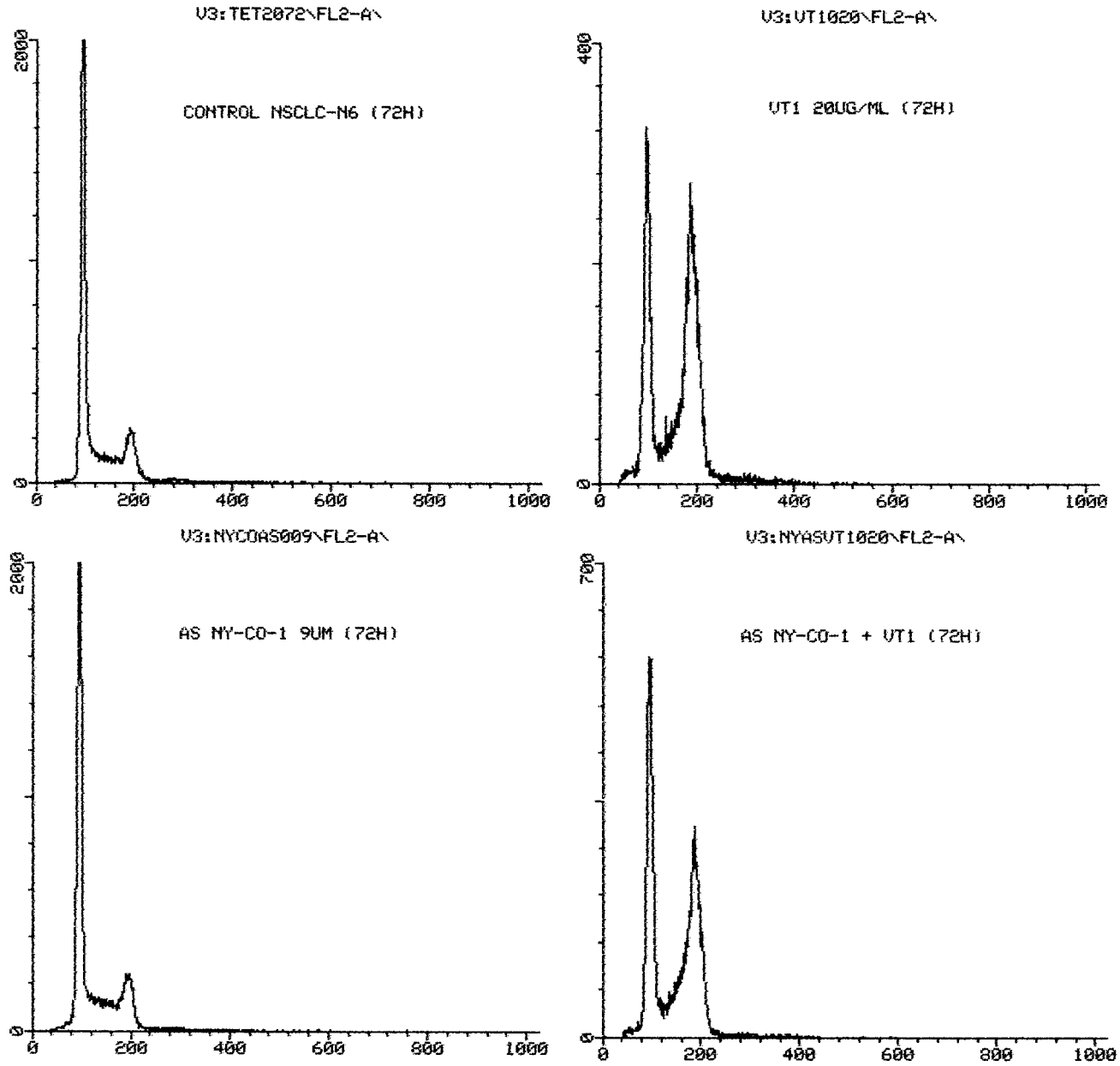


FIGURE 10 – Histograms of the DNA of NSCLC-N6 cells cultured for 72 hr in the absence (control) and presence of VT1 (20 μg/mL) and/or NY-CO-1 antisense oligonucleotide (9 μM).

results are in agreement with precedent studies in our laboratory[20] and show a "differentiation pattern" with particular production of VT1-treated tumor cells probably linked with genetic modifications.

Characterization of differentially expressed genes

Differential display. DD of mRNA present in cells treated with 7 μg/mL VT1 vs. nontreated cells is illustrated in Figure 6*a,b*. Bands G4 1 and 2, which resulted from the amplification lane with HAP4 and $HT_{11}G$ primers, were present in treated cells but had a very weak signal in tumor cells. A partial sequence of band 1 and 2 showed that they were identical. In fact, it is usual in differential display for pairs of adjacent bands to represent the paired cDNA strands separated on denaturing DD gels.[21] To confirm overexpression, part of the cDNA was isolated, reamplified and used as a probe in an RPA assay.

RPA analysis. RPA analysis of N6 cells with or without VT1 treatment revealed that the expression of fragment 1–2 was clearly induced by VT1 treatment (Fig. 7*a,b*). These findings were in agreement with the differential display results and confirm the overexpression of fragment 1–2 after arrest of N6 cells proliferation. Moreover, RPA analysis was performed with total RNA extracted from tumor samples developed after subcutaneous xenograft of N6 cells in nude mice and treatment with various concentrations of VT1. Figure 8*a,b* shows the results of RPA analysis from tumor samples of mice treated intravenously with 60 mg/kg VT1 or intraperitoneally with 100 mg/kg and 150 mg/kg VT1. These preliminary *in vivo* results confirmed *in vitro* observations.

Identification of fragment 1–2. Submission of fragment 1–2 against GenBank databases showed that it was homologous to a recently characterized cDNA, NY-CO-1 (New-York Colon Antigen 1), a human antigen of unknown function cloned by immu-

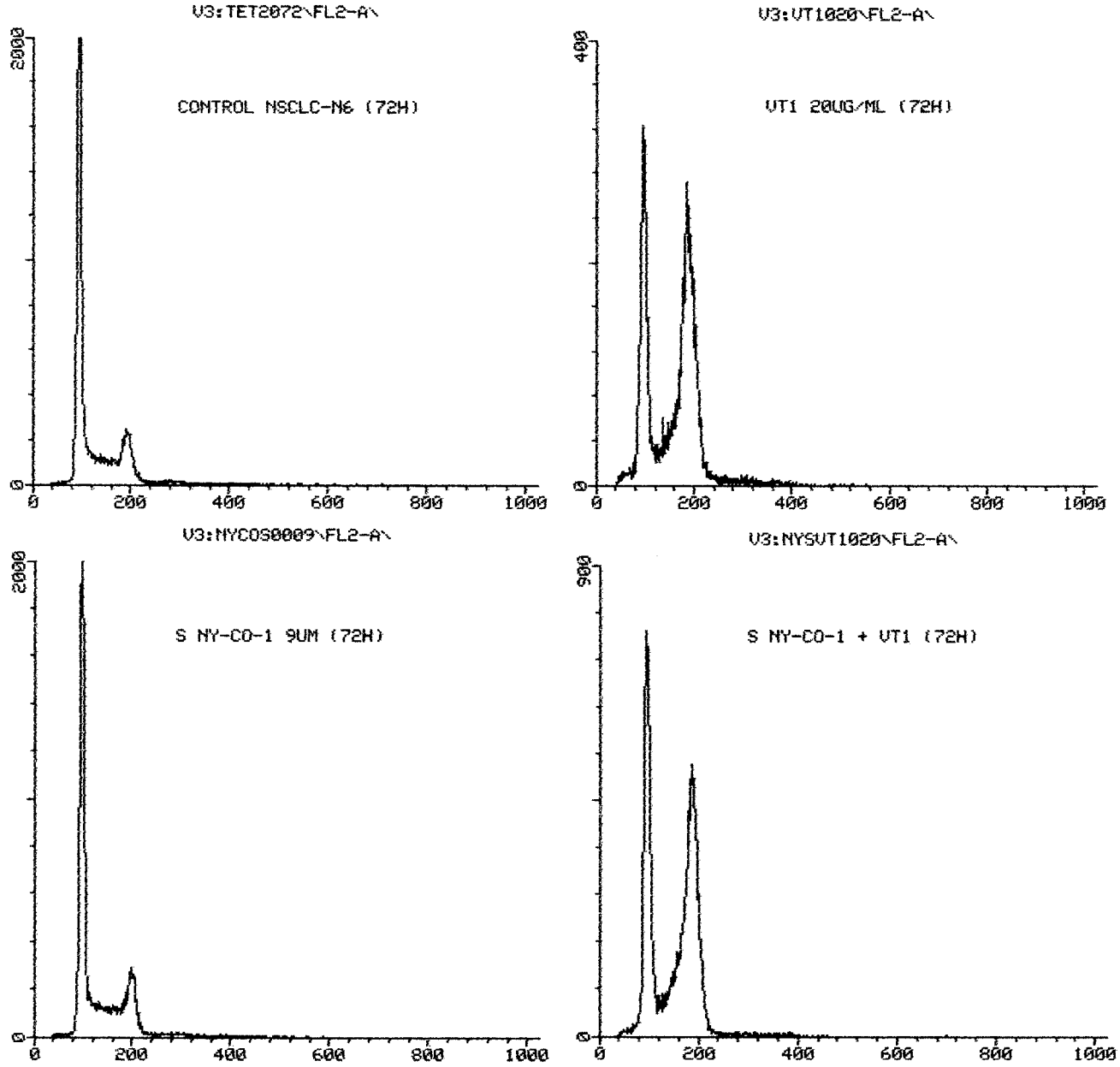


FIGURE 11 – Histograms of the DNA of NSCLC-N6 cells cultured for 72 hr in the absence (control) and presence of VT1 (20 μg/mL) and/or NY-CO-1 sense oligonucleotide (9 μM).

noscreening of colon cancer cDNA expression libraries with autologous sera (GenBank AF039687).[22] Analysis of deduced amino acid sequence indicated the presence of a nuclear targeting signal.

Antisense treatment. The antisense strategy was performed to investigate the implication of NY-CO-1 in the arrest of N6 cell proliferation after treatment with VT1. This method was used to test the hypothesis that NY-CO-1 inhibition of expression, in association with treatment of VT1, blocks arrest of cell proliferation. Figure 9 shows that antisense treatment of N6 cells accelerated growth proliferation. This effect was dose-dependent and apparent only after 24 hr of treatment, probably because the half-life of nonmodified oligonucleotide is very short in these culture conditions. Thus, antisense inhibition of NY-CO-1 accelerates N6 proliferation. The results for the activity of 9 μM of antisense oligonucleotide on the distribution of N6 cells in the cell cycle are presented in Figure 10. The treatment of cells with AS-NY-CO-1 alone did not modify the distribution profile of N6 cells after 72 hr treatment compared with control cells (Fig. 10*a*). Conversely, in the presence of antisense oligonucleotide, the proliferation arrest and blockade in G1 phase (Fig. 10*c*) induced by VT1 was decreased (Fig. 10*d*). This was not a cytotoxic effect of the oligonucleotide since the control "sense" oligomer (for the same dose) did not modify the N6 cell distribution profile in the cell cycle (Fig. 11). Thus, the inhibition of NY-CO-1 after induction by treatment with VT1 stopped proliferation arrest and blockade in G1 phase normally apparent with VT1 alone.

DISCUSSION

Carcinogenesis leads to disturbance in the expression of a group of genes, oncogenes and anti-oncogenes. Although a large number of these genes have been identified, many are still unknown.

Moreover, experimental induction of tumor cell death has shown that the cells still possess a genetic system, allowing them to die in any stimulation conditions. In this context, an attempt was made here to relate a genetic effect to the antiproliferative activity of VT1.

VT1 activity was first defined in our *in vitro* experimental model, the N6 line, which showed that the cytostatic activity of VT1 on the N6 line caused no immediate cell death. This activity was due to a blockage in the G1 phase, which was irreversible and associated with important morphological changes. These results are to compare with those reported for the bistramide family in previous studies. In-depth study of this family has revealed an activity-inducing terminal differentiation both *in vitro* and *in vivo*.[17]

On the basis of these data, the analysis begun with VT1 was developed further by studying the genetic variations to the treatment. This approach was based on the differential display strategy developed in our laboratory.[13] This study allowed selection of the 1–2 fragment. The differential display expression of this messenger was confirmed both *in vitro* and *in vivo*. At this stage, a consultation of data banks showed that the messenger was characterized in 1998 by a New York team during systematic screening of colonic tumor antigens by an immunological method.[22]

The gene from which NY-CO-1 is derived is located in 14q22.[23] As it has been reported, the 14q22 zone was disturbed in some gastrointestinal[24] and renal tumors,[25,26] as well as in acute lymphocytic leukemias.[27] Thus, this gene is located in a zone of a human genome that has already been implicated in carcinogenesis of different cell types, so that the role of NY-CO-1, initially described in colonic cancer, would seem to concern a larger number of cell types. Our own studies have revealed its implication in lung carcinogenesis.

These different data led us to try to define the function of this messenger. NY-CO-1 is induced after VT1 treatment, thus after induction of arrest of N6 cell proliferation. The attempt to define its function was made in this context.

The antisense strategy was used to define the *NY-CO-1* function and confirm its possible relation with the arrest of tumor-cell proliferation. For this purpose, two types of antisense sequences were selected. First, a deoxyribooligonucleotide (oligonucleotide) sequence was used according to the criteria of Thompson *et al.*[19] Studies of the cell-growth profile of the N6 line after treatment with antisense sequences were the same as those used for the analysis of VT1 activity.

The results obtained with cell-growth kinetics after direct treatment by the oligonucleotide showed that test cells underwent accelerated growth 24 hr later compared with untreated cells or those treated with a control oligonucleotide. These initial results led us to envisage an analysis of the antisense action profile at the level of the cell cycle. As NY-CO-1 was up-regulated after VT1 treatment, the cells were treated simultaneously with VT1. This direct treatment of the cells showed a modification of the cell distribution profile in the cycle.

The distribution profile of cells under the action of the antisense oligonucleotide alone was not really modified compared with controls. In fact, since N6 expressed NY-CO-1 messenger only slightly, its inhibitory effect was poor. Conversely, in the presence of VT1, the distribution profile of cells changed compared with those treated by VT1 alone. The blockade induced by VT1 alone in the G1 phase was lower, as indicated in flow cytometry by a reduction in the G1 peak of polyploidic cells (4n chromosomes). Oligonucleotide activity played a "recruiting" role for cells in the cycle and was competitive with VT1 activity.

In conjunction with this study, the choice of a control sequence (sense sequence) allowed us to validate our study concerning the functional approach of NY-CO-1.

In terms of the antisense strategy, all of the results concerning the functional approach of NY-CO-1 showed that NY-CO-1 plays a role in stopping N6 cell proliferation after induction subsequent to treatment. An inhibition of NY-CO-1 function by an antisense sequence returns the cells into proliferation mode.

In conclusion, these results indicate that the new messenger, NY-CO-1, is involved in stopping the proliferation of the N6 line after VT1 treatment. The action of VT1 induces genes whose function is to produce this effect. The induction of cell proliferation, differentiation and programmed death leads to a cascade of events in all cell compartments. NY-CO-1 contributes to theses processes and thus could be considered as a new pharmacologic target gene in tumor therapy.

REFERENCES

1. Matter J, Volm M. Resistance mechanisms in human lung cancer. Inv Metastasis 1995;15:81–94.
2. Harwood K. Non-small cell lung cancer: an overview of diagnosis, staging and treatment. Sem Oncol Nursing 1996;12:285–94.
3. American Society of Clinical Oncology. Clinical practice guidelines for the treatment of unresectable non-small-cell lung cancer. J Clin Oncol 1997;15:2996–3018.
4. Roussis V, Ampofo SA, Wiemer DF. A phenylated benzoic-acid derivative from the leaves of piper toboganum phytochemistry. 1990; 24:1787.
5. Roussakis C, Gratas C, Audouin AF, Le Boterff J, Dabouis C, Andre MJ, et al. Study of in vitro drug sensitivity on a newly established cell line from a primary bronchial epidermoid carcinoma of human origin (NSCLC-N6). Anticancer Res 1991;11:2239–44.
6. Riou D, Roussakis C, Robillard N, Biard JF, Verbist JF. Bistramide A-induced irreversible arrest of cell proliferation in a non-small-cell lung bronchopulmonary carcinoma is similar to induction of terminal differentiation. Biol Cell 1993;77:261–4.
7. Bourgougnon N, Roussakis C, Kornprobst JM, Lahaye M. Effects in vitro of sulfated polysaccharide from Schizymenia dubyi on a non-small-cell bronchopulmonary carcinoma line (NSCLC-N6). Cancer Lett 1994;85:87–92.
8. Bergé JP, Bourgougnon N, Carbonnelle D, Le Bert V, Tomasoni C, Durand P, et al. Antiproliferative effects of an organic extract from the marine diatom Skeletonema costatum against a non-small-cell bronchopulmonary carcinoma line (NSCLC-N6). Anticancer Res 1997;17: 2115–20.
9. Yonish-Rouach E. The p53 tumor suppressor gene: a mediator of a G1 growth arrest and of apoptosis. Experientia 1996;52:1001–7.
10. Korsmeyer SJ. BCL-2 gene family and the regulation of programmed cell death. Cancer Res 1999;59 (Suppl.):1693–700.
11. Thornberry NA, Lazebnik Y. Caspases: enemies within science. 1998; 281:1312–6.
12. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. Science 1998;281:1305–8.
13. Liang P, Pardee AB. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 1992;257: 967–71.
14. Mossmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
15. Vindelov LL. Flow microfluorometric analysis of nuclear DNA in cell forms solid tumors and cell suspensions. A new method for rapid isolation and staining of nuclear DNA. Virchows Archi B Cell Pathol 1977;24:227–42.
16. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156–9.
17. Riou D, Colliec-Jouault S, Pinczon Du Sel D, Bosch S, Siavoshian S, Le Bert V, et al. Antitumor and antiproliferative effects of a fucan extracted from ascophyllum nodosum against a non-small-cell bronchopulmonary carcinoma line. Anticancer Res 1996;16:1213–8.
18. Bösch S, Siavoshian S, Jacquot C, Tomasoni C, Dabouis G, Elanbalouss Y, et al. Correlation between multidrug resistance and the degree of differentiation of non-small cell bronchopulmonary carcinoma (NSCLC-N6) in vitro and in vivo. Anticancer Res 1997;17: 4595–8.
19. Thompson ME, Jensen RA, Obermiller PS, Page DL, Holt JT. Decreased expression of BRCA1 accelerates growth and is often present during sporadic breast cancer progression Nature Gen 1995;9:444–50.
20. Roussakis C, Robillard N, Riou D, Biard JF, Pradal G, Piloquet P, et

al. Effects of Bistramide A on a non-small-cell bronchial carcinoma line. Cancer Chemother Pharmacol 1991;28:283–92.
21. Martin KJ, Kwan CP, O'Hare MJ, Pardee AB, Sager R. Identification and verification of differential display cDNAs using gene-specific primers and hybridization arrays. Biotechniques 1998;24:1018–26.
22. Scanlan MJ, Chen YT, Williamson B, Gure AO, Stockert E, Gordan JD, et al. Characterisation of human colon cancer antigens recognised by autologous antibodies. Int J Cancer 1998;76:652–8.
23. Carbonnelle D, Liehr T, Jacquot C, Masson D, Lustenberger P, Denis MG, et al. Assignment of the serologically defined colon cancer antigen 1 (SDCCAG1) to human chromosome 14sq22 by in situ hybridization. Cytogen Cell Gen 1999;86:3–4.
24. El Rifai W, Slormo-Rikaka M, Miettinen M, Knuutila S, Anderson LC. DNA copy number losses in chromosome 14: an early change in gastrointestinal stromal tumours. Cancer Res 1996;56:3230–3.
25. Kovacs G, Frisch S. Clonal chromosome abnormalities in tumor cells from patients with renal carcinomas. Cancer Res 1989;49:651–9.
26. De Jong B, Oosterhuis JW, Idenburg VJ, Castedo SM, Dam A, Mensink HJ. Cytogenetics of 12 cases of renal adenocarcinoma. Cancer Genet Cytogenet 1988;30:53–61.
27. Lai JL, Fenaux P, Pollet JP, Estienne MH, Savary JB, Huart JJ, et al. Acute lymphocytic leukaemia with 9p anomalies. A report of four additional cases and review of the literature. Cancer Genet Cytogenet 1988;33:99–109.