Vinorelbine Induces β3-Tubulin Gene Expression through an AP-1 Site

JENNIFER SAUSSEDE-AIM[1], EVA-LAURE MATERA[1], STEPHANIE HERVEAU[1], JEAN-PIERRE ROUAULT[2], CHRISTIANO FERLINI[3] and CHARLES DUMONTET[1]

[1]Inserm U590, Laboratoire de Cytologie Analytique, Université Claude Bernard Lyon 1, Lyon;
[2]IGFL UMR 5242 CNRS-INRA UCBL ENS, Lyon, France;
[3]Laboratory of Antineoplastic Pharmacology, Department of Obstetrics and Gynecology, Catholic University of the Sacred Heart, Rome, Italy

Abstract. *Although the correlation between β3-tubulin expression level in tumors has been well correlated with clinical outcome in patients receiving microtubule-targeted agents, the regulation of this protein remains poorly understood. Recently, new insight of regulatory processes was offered with the cloning of the gene promoter. In this study β3-tubulin gene expression was induced in response to exposure to various antimicrotubule agents such as vinorelbine and paclitaxel. The exploration of the β3-tubulin gene promoter by successive deletions followed by site-directed mutagenesis led to the localization of a vinorelbine-responsive element containing an activator-protein 1 (AP-1) site. Among the various antimicrotubule agents tested, it appeared that the implicated AP-1 site was activated only after exposure to vinorelbine. This study confirms the inducible nature of the β3-tubulin gene promoter.*

Tubulin is the basic constituent of microtubules which play a role in multiple essential functions for cell life, such as motility, shape maintenance and cell division. During mitosis, polymerization of αβ-tubulin dimers permits the growth of microtubules which form the mitotic spindle. This property has made microtubules and tubulin a target of choice for anticancer drugs. Antimicrotubule agents are divided into two groups according to their stabilizing or destabilizing mode of action at high concentration. Antimicrotubule agents are widely used in the treatment of breast and lung carcinomas. However, the main limitation in the use of such compounds arises from the development of resistance. Among the processes implicated in the decreased response to drugs, the multidrug resistance (MDR) phenotype has been observed *in vitro*, but less well documented *in vivo* (1). Indeed, it has been demonstrated that efflux pumps, especially P-glycoprotein, are not consistently overexpressed in cases of resistance to antimicrotubule drugs (2). This has suggested the presence of concomitant resistance mechanisms in numerous non-responsiveness cases. The enhanced expression of β3-tubulin isotype expression has been correlated to reduced response to antimicrotubule agents, in lung (3), breast (4), prostate (5) and ovarian carcinomas (6). Moreover, the specific implication of β3-tubulin in the response to antimicrotubule agents has been shown in non-small cell lung cancer by using specific siRNA against this isotype (7). However, it has not been reported whether exposure to these antimicrotubule agents in itself influences β3-tubulin content in tumors.

Despite the well-established correlation between high levels of β3-tubulin expression in tumors and outcome in patients receiving microtubule-targeted agents, the regulation of this protein remains unclear. During the 1980s, the model of an autoregulatory mechanism was proposed to explain the regulation of β-tubulin peptides (8). This was based on the observation that a decrease of the free tubulin pool is necessary for the induction of tubulin synthesis. Indeed, eukaryotic cells exploit mRNA instability as a means to precisely control the level of the monomer tubulin pool (9). This autoregulatory mechanism appears to be a general process explaining expression regulation of all tubulins without isotype distinction. In addition, the presence of the MREI (met-arg-glu-ile) amino acids sequence, which is likely to be both sufficient and necessary to activate the regulatory mechanism in all β-tubulin isotypes, supports the hypothesis that the autoregulatory mechanism is a common system to control β-tubulin expression level (10). The transcription factors involved in basal cell expression of β3-tubulin have not been determined. Conversely the induction of β3-tubulin has been demonstrated by several authors using

Correspondence to: Jennifer Saussede-Aim, Laboratoire de Cytologie Analytique, Faculté de Medecine Rockefeller, 8 Avenue Rockefeller, 69008 Lyon, France. Tel: +33 478777236, Fax: +33 478777088, e-mail: jennifer.saussede-aim@laposte.net

Key Words: Antimicrotubule agents, β3-tubulin, regulation, AP-1.

different stimuli. In these cases, the regulation has been shown to involve a transcriptional mechanism. β3-Tubulin expression appears to respond to hypoxia through a hypoxia-inducible factor 1 (HIF-1) site located on the 3' flanking region (11). Moreover, our group has recently demonstrated that estradiol regulates β3-tubulin expression *via* an estrogen receptor (12). Here, the regulation of β3-tubulin expression induced by antimicrotubule agents through a gene response element in a model of breast cancer is reported.

Materials and Methods

Cell culture. Human breast carcinoma MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (200 units/ml) and streptomycin (200 μg/ml). The cells were cultured at 37˚C in a humidified atmosphere and 5% CO_2. The cells were treated with 1 μM vinorelbine (Pierre Fabre Medicament, Castres, France), vinblastine (Lilly, Suresnes, France), or colchicine (Sanofi-Aventis, Paris, France), or by 1 nM paclitaxel (Bristol-Myers Squibb, Rueil-Malmaison, France) dissolved in phosphate-buffered saline.

Quantitative real-time PCR. The total mRNA was extracted using Trizol reagent (Invitrogen, Cergy Pontoise, France) following the manufacturer's instructions. Two micrograms were converted into cDNA by Moloney leukaemia virus reverse transcriptase (Invitrogen) for one hour at 37˚C as described in the manufacturer's manual. The cDNA levels were normalized to the expression of the 18S ribosomal gene using the pre-developed TaqMan assay reagents control kit (Applied Biosystem, Foster City, Canada) in a LightCycler thermal cycler (Roche, Meylan, France) with denaturation/annealing at 95˚C for 15 s and extension at 60˚C for 1 min. Specific mRNA levels for β3-tubulin, β1-tubulin and the total β-tubulin gene were quantified by real-time PCR in a final reaction volume of 6.67 μl containing forward and reverse primers (300 nM each), $MgCl_2$ (2 mM), deoxyribonucleotide triphosphates (500 μM) and LC-FastStart DNA Master SYBRGreen (0.67 μl) (Roche). The PCR conditions were denaturation at 95˚C for 15 s, annealing at 58˚C for 5 s and extension at 64˚C for 10 s. The sequences of the primers are presented in Table I. Then results were analyzed using Relquant software (Roche) as indicated in the user's manual and results are given as the mean value of three independent experiments expressed in arbitrary units using unexposed cells as reference.

Promoter cloning and deletion. The 2.3 kb promoter region upstream of the β3-tubulin gene (gene ID: 10381) coding the β3-tubulin protein and containing the putative transcription start (11) was cloned into a pGL3 basic plasmid (Promega, Charbonnières, France) with the luciferase gene as reporter using *Xho*I and *Hind*III. This plasmid construct was named p5'tubβ3. Various lengths of DNA fragments upstream of the β3-tubulin gene were amplified by PCR and cloned into the pGL3 basic plasmid. The sequences of the primers used for amplification of the deletion constructs are summarized in Table II. PCR were performed by initial denaturation for 2 min at 94˚C, 35 cycles of denaturation for 1 min at 94˚C, annealing for 1 min at 60˚C and extension for 1 min at 68˚C, followed by a final extension for 7 min at 68˚C. Each construct was entirely sequenced by Cogenics (Meylan, France).

Table I. *Primer sequences used for quantitative PCR.*

Gene	Primer	Sequence
β3-Tubulin	Lβ4 F	ggcctttggacatctcttca
	Lβ4 R	cggtcgggatactcctca
β1-Tubulin	LM40 F	tgccccatacataccttgag
	LM40 R	ggaacatatttgccacctgt
β-Tubulin	βtub-159 F	cacaggcagttaccatggag
	βtub-322 R	gtctgaagatctggccgaag

Site directed mutagenesis. Mutant promoter reporters were generated by site-directed mutagenesis on the template p5'tubβ3 with a QuickChange™ site-directed mutagenesis kit (Stratagene, Massy, France) by using primers: 760mp5'tubβ3F (gggagaccggac ggtcctacagccttaagcccggc) and 760mp5'tubβ3R (gccgggcttaag gctgtaggaccgtccggtctccc) for the deletion named 760mp5'tubβ3, and 764mp5'tubβ3F (gggagaccggacggtgaggcagccttaagcccggc) and 764mp5'tubβ3R (gccgggcttaaggctgcctcaccgtccggtctccc) for the deletion named 764mp5'tubβ3. DMSO (5%) was added to the PCR mix due to the high GC content of the amplified region and PCR was performed by initial denaturation for 1 min 30 at 95˚C, followed by 18 cycles of denaturation/annealing for 1 min at 95˚C and extension for 8 min at 68˚C. XL-gold® ultracompetent cells (Stratagene) were then transformed with the PCR products. Each construct was entirely sequenced by Cogenics.

Measurement of promoter activity using a luciferase assay. MCF-7 cells were transfected by Lipofectin reagent (Invitrogen) following the manufacturer's instructions. Briefly the cells were seeded into 12-well plates at a density of 75,000 cells per well the day before transfection. To correct for different efficiency of transfection, Renilla luciferase plasmid pRL-SV40 was co-transfected into the cells with each pGL3 basic construct. The cells in each well were cultured with 1 ml of DMEM. The MCF-7 cells were transfected by 14 ng pRL-SV40 and 1 μg pGL3 basic promoter construct. After 6 hours of transfection the cells were incubated with drugs for 48 hours. The cells were harvested for determination of Renilla and firefly luciferase activities by the Dual Luciferase reporter assay system (Promega). Duplicate assays were performed for each condition. The results were expressed as average relative firefly luciferase activity normalized with Renilla luciferase activity. Each experiment was performed three times independently.

Results

β3-Tubulin mRNA induction by vinorelbine. After 24-hour exposure to 1 μM vinorelbine, the β3-tubulin mRNA level was increased (4-fold) and similar levels were found at 48 and 72 hours' exposure time (Figure 1). Conversely, the mRNA level of β1-tubulin, the most abundant isotype, was not enhanced following vinca exposure. Due to the clinical relevance of β3-tubulin expression, this isotype was studied exclusively in the subsequent experiments. Total β-tubulin mRNA levels were increased 2-fold after 4 and 24 hours of

Table II. *Primer sequences used for promoter constructs.*

Amplicon name	Primer name	Sequence	Size (bp)
622p5'tubβ3	622p5'tubβ3 F	tggaggctagcgggccatcagccgactccta	1720
	131pGL R	gctctccagcggttccatcttccagcggat	
1104p5'tubβ3	1104p5'tubβ3 F	agttcgctagcgctcattttgtgaagtgaatgc	1238
	131pGL R	gctctccagcggttccatcttccagcggat	
1696p5'tubβ3	1696p5'tubβ3 F	cctgggctagcctttacctacctccccca	646
	131pGL R	gctctccagcggttccatcttccagcggat	
2225p5'tubβ3	2225p5'tubβ3 F	cgtccgctagcgcccgccagacgcgcccagt	117
	131pGL R	gctctccagcggttccatcttccagcggat	
693p5'tubβ3	693p5'tubβ3 F	tgactgctagcctcaggaactgggagc	1757
	131pGL R	gctctccagcggttccatcttccagcggat	
810p5'tubβ3	810p5'tubβ3 F	tgcccgctagctgcaaaacttcttccc	1640
	131pGL R	gctctccagcggttccatcttccagcggat	
910p5'tubβ3	910p5'tubβ3 F	aggcagctagcccccagcagcagcca	1540
	131pGL R	gctctccagcggttccatcttccagcggat	

Various lengths of β3-tubulin promoter were amplified by PCR using p5'tubβ3 as a template and cloned into a pGL3 basic plasmid.

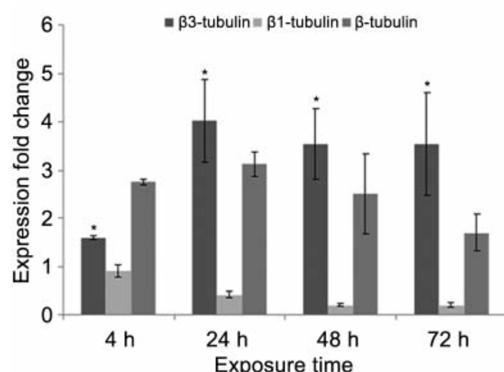

Figure 1. *Time-course analysis of the expression of various β-tubulin genes after exposure to 1 μM vinorelbine. mRNA levels were measured by RT-quantitative PCR in MCF-7 cells. Bars and error bars represent means of three experiments and standard deviation. *p<0.05 when compared to unexposed cells, determined by Student's t-test.*

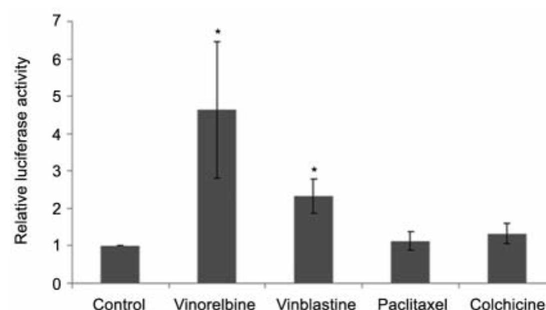

Figure 2. *β3-Tubulin promoter activity in MCF-7 cells after exposure to antimicrotubule agents. The entire promoter sequence of the β3-tubulin gene was cloned upstream of a luciferase gene reporter (p5'tubβ3). Transfected cells were then exposed to 1 μM vinorelbine, vinblastine or colchicine, or 1 nM paclitaxel for 48 hours. Luciferase activity in exposed cells was normalized with the corresponding luciferase activity of unexposed cells (control). Bars and error bars represent means of three experiments and standard deviation. *p<0.05 when compared to untreated cells, determined by Student's t-test.*

exposure and then decreased at 48 and 72 hours. The induction of β3-tubulin seemed to be partially compensated by a decrease of β1-tubulin, indicating that a regulatory system to control β-tubulin levels might exist.

A similar inducible effect on β3-tubulin mRNA expression was also observed with vinblastine, another vinca alkaloid agent, as well with the antimicrotubule agents colchicine and paclitaxel (data not shown).

β3-Tubulin regulation via its promoter. To verify whether antimicrotubule agents regulate β3-tubulin gene expression *via* its promoter, the plasmid p5'tubβ3 containing the promoter region of the β3-tubulin gene cloned upstream of a luciferase gene was transfected into the MCF-7 cells before exposure to these agents. The data presented in Figure 2 clearly show that the luciferase activity, corresponding to the promoter activity, increased 2- to 4-fold following the 48 hours of vinca exposure. Conversely, paclitaxel and colchicine did not affect promoter activity, indicating that the 2.3 kb region upstream of the β3-tubulin gene contained an

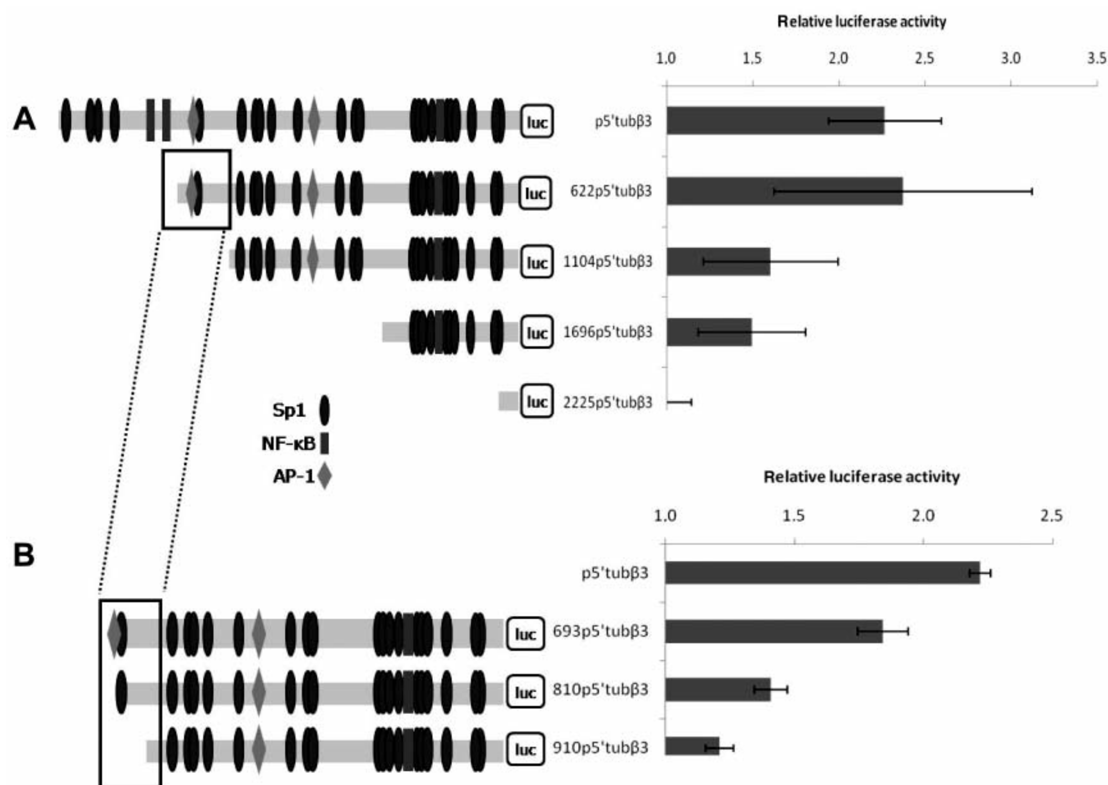

Figure 3. *Effect of β3-tubulin promoter deletions in MCF-7 cells exposed to vinorelbine. Entire promoter sequence of β3-tubulin gene and deleted fragments were cloned upstream of a luciferase gene (luc) reporter and transfected into MCF7 cells. Transfected cells were exposed to 1 μM vinorelbine for 48 hours. Luciferase activity in vinorelbine-treated cells was normalized with the corresponding luciferase activity of unexposed cells. A: Successive deletions of the promoter. B: Deletions within the 622-1104 bp segment of the promoter. Transcription factor binding sites were identified by MatInspector analysis of β3-tubulin promoter. Bars and error bars represent means of three experiments and standard deviation.*

enhancer binding site for vinca-responsive elements. Vinorelbine, which induced the strongest response among the compounds tested, was chosen for the subsequent exploration of the regulatory mechanism.

Mapping of enhancer sequences activated by vinorelbine. The binding sites for transcription factors involved in β3-tubulin expression appeared to be located in the promoter region. In order to precisely map these sequences, the promoter was successively deleted before transfection into the MCF-7 cells (Figure 3A). No significant change of luciferase activity was observed when the first 622 bp fragment was removed. However, once the segment between 622 and 1104 bp was eliminated, the response to vinorelbine exposure decreased 50% , indicating that this part contained the regulating elements involved in the vinorelbine-response. A similar effect was observed when the fragment between 1696 and 2225 bp was removed. The deletion of the portion 1104 to 1696 bp did not induce a modification in the luciferase activity, showing that it did not contain major sites involved in the response.

A second series of deletions was performed in order to investigate specifically the segment 622 to 1104 bp which was found to contain the main regulatory sites. The promoter activity decreased gradually with the extent of the deletions (Figure 3B). Nevertheless, the most important reduction of the response to vinorelbine was detected when the segment between 693 and 810 bp was removed. This decrease corresponded to the decrease obtained when the entire fragment 622 to 1104 bp was deleted, thus suggesting that the major binding sites for the response element, activated by vinorelbine, were located in this portion.

Role of the AP-1 site in vinorelbine-induced β3-tubulin expression. The putative binding sites on the promoter fragment between 693 and 810 bp were determined by *in silico* analysis by Matinspector software (Genomatix), and the AP-1 site, located between 757 and 767 bp, was then mutated. Two previously described mutations of this site, 760mp5'tubβ3 (13) and 764mp5'tubβ3 (14) were introduced to study the role in the vinca response. When the AP-1 site was mutated at only one nucleotide (764mp5'tubβ3), the

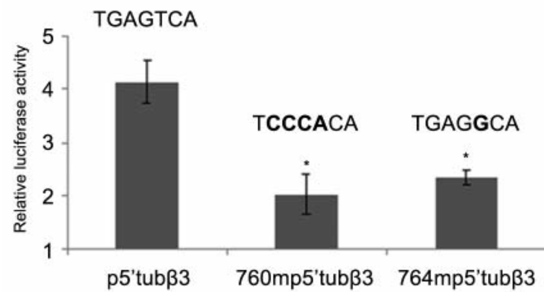

Figure 4. *Effect of β3-tubulin promoter mutations in MCF-7 cells exposed to vinorelbine. The entire β3-tubulin promoter was cloned upstream of a luciferase gene reporter and then mutated at position 760 (760mp5'tubβ3 construct: four mutated nucleotides) or 764 (764mp5'tubβ3 construct: one mutated nucleotide) and transfected into MCF7 cells. Transfected cells were exposed to 1 μM vinorelbine for 48 hours. Luciferase activity in vinorelbine-exposed cells was normalized with the corresponding luciferase activity of unexposed cells. Bars and error bars represent means of three experiments and standard deviation. Mutated nucleotides are written in bold over bars. *$p<0.05$ when compared to cells transfected with complete promoter (p5'tubβ3) determined by Student's t-test.*

response to the vinorelbine exposure decreased by 50% (Figure 4). The same result was obtained when the AP-1 site possessed 4 mutated nucleotides (760mp5'tubβ3). The decline of the promoter activity due to the mutations was similar to the decrease observed when the 693 to 810 bp fragment was removed, thus confirming that the AP-1 binding site was involved in the vinorelbine-induced response.

Discussion

For the first time, the modulator role of antimicrotubule drugs on β3-tubulin gene expression was demonstrated. The various antimicrotubule agents were applied at concentrations known to trigger signalling pathways, including activation of c-jun *N*-terminal kinase (15), nuclear factor-κB (16) and phosphorylation of bcl-2 (17). Indeed the present report demonstrated that four different antimicrotubule drugs induced β3-tubulin mRNA in a breast cancer model. These data provided evidence of the essential role of the β3-tubulin promoter especially in the response induced by vinorelbine. Until now, the promoter activity of the region upstream of the human β3-tubulin gene has been characterized without description of any regulatory sites (11). The inducible nature of β3-tubulin gene expression was also demonstrated after estradiol exposure (12). The identification of the site involved in the vinorelbine response on the promoter region confirmed the promoter activity of the cloned segment and the successive deletions followed by site-directed mutations showed the involvement of an essential AP1-binding site in the vinorelbine response.

AP-1 sites are known to bind preferentially homodimeric or heterodimeric proteins made up by the association of Jun and Fos proteins. Moreover, these proteins have been shown to be involved in the transduction pathway of vinca alkaloids (18). However, no binding of Jun (c-jun, junB, junD) or Fos (c-fos, FosB, Fra-1, Fra-2) proteins to the AP-1 site in the β3-tubulin promoter was observed by chromatin immunoprecipitation (data not shown). The identity of the transcription factors recruited for the vinorelbine response therefore remains to be elucidated. The investigation could now be oriented to transcription factors able to bind AP-1 sites, but not belonging to Jun and Fos families, such as c-jun dimerization protein 2 (JDP2), Jun activation domain-binding protein 1 (JAB1), or activating transcription factor (ATF-2) (19). For example, ATF-2 has been reported to bind the AP-1 site located on the human urokinase gene promoter (20).

The binding of a transcription factor other than a conventional Jun/Fos heterodimer on the identified AP-1 site is intriguing because the phosphorylation of c-jun in response to exposure to antimicrotubule agents, and especially vinca alkaloids, is well documented (21). Nevertheless, antimicrotubule agents are able to trigger a variety of signal transduction pathways such as those involving the Bcl-2 gene family (22) and caspase family (23). Numerous pathways have been suggested to explain the induction of apoptosis, but it appears that they are triggered according to the kind of antimicrotubule agent used (24).

A variety of pathways is consistent with the different responses observed with the different agents. Although all the tested drugs in the present study increased β3-tubulin gene expression level, only the two vinca alkaloids were able to activate promoter activity, confirming that different pathways govern this induction. Among the binding sites present on the β3-tubulin promoter, at least one is clearly identified as a vinorelbine-responsive element. One or several other sites located between 1696 and 2225 bp complete the response to vinorelbine. Indeed when this part was removed, the induction by vinorelbine was completely abolished. Conversely in the case of colchicine and paclitaxel, responsive elements were not found in the β3-tubulin promoter. Taken together, the evidence of these data tend to indicate that the β3-tubulin promoter contains regulatory elements specifically activated by a particular class of antimicrotubule drugs.

β3-Tubulin gene-inducing properties of the four different antimicrotubule agents used in breast cancer model might have relevant applications in the clinic. Indeed the increased expression of β3-tubulin mRNA was maintained throughout 72 hours of exposure to antimicrotubule agents. This data indicated that enhanced expression of β3-tubulin could result from treatment in patients receiving these agents. This hypothesis is strengthened by the demonstration that a high basal level of β3-tubulin was not

sufficient to obtain a decreased response to antimicrotubule agents in prostatic carcinoma cells (25), suggesting that a high basal level of tubulin may not be associated with reduced sensitivity to antimicrotubule agents in all cases. This new insight of β3-tubulin regulation raises the question of the utility of β3-tubulin in predicting the response to these drugs before the administration of a microtubule-targeted treatment (26, 27). Moreover the correlation between an elevated level of β3-tubulin and a decreased response to antimicrotubule agents could be the result of the emergence of more aggressive tumors, as suggested by reports identifying β3-tubulin as a prognostic, rather than as a predictive, factor (28).

In conclusion, this study provides the first data of specific regulation of a given tubulin isotype in response to anticancer agents. These data also raise the intriguing possibility that exposure to microtubule-targeted drugs in the clinic could contribute to overexpression of β3-tubulin, thereby enhancing acquired resistance to these agents.

Acknowledgements

J. Saussede-Aim was supported by a fellowship from the Ligue Contre le Cancer (Comité de la Loire). We thank Dr. L.P. Jordheim for his critical review of the manuscript.

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Received March 11, 2009
Revised May 24, 2009
Accepted June 11, 2009