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Research paper

The effect of combining antitubulin agents on differentiated and undifferentiated human colon cancer cells

G Carles, D Braguer, G Sabeur and C Briand
UPRES A-CNRS 6032, Faculty of Pharmacy, 27 Bd Jean Moulin, 13005 Marseille Cedex 5, France.
Tel/Fax: (+33) 4 91 78 20 24.

The cytotoxicity of sequential combinations of a taxoid [paclitaxel (TAX) or docetaxel (TXT)] with a vinca alkaloid [vinorelbine (NVB)] was compared in differentiated and undifferentiated HT29-D4 cells. Agents were titrated from low doses inducing no modification of microtubule network to high doses corresponding to the clinically relevant concentrations that block mitosis. For undifferentiated cells, the sequential combination NVB/TAX was more efficient than TAX/NVB (22% cell survival versus 37% for 5 nM TAX and NVB). Surprisingly, we successively obtained synergism for low doses of both compounds [NVB (1–5 nM) and TAX (1–15 nM)], then additivity and finally antagonism when one of the compounds was at the concentration inducing mitotic block. The three patterns of results were also obtained with NVB/TXT combinations. For the synergistic combinations at the lowest concentrations, cytotoxicity occurred by apoptosis following mitosis. For differentiated cells, the most cytotoxic combinations were 1 μM TAX or TXT for 3 days followed by 1 μM NVB for 3 days, and 0.75 nM TAX or TXT for 9 days followed by 1 μM NVB for 3 days, the latter producing synergistic effects. Cytotoxicity occurred by apoptosis for the two states of differentiation. Major differences depending on cell phenotype were demonstrated: low sensitivity of differentiated cells to antitubulin agents and the difference in apoptotic pathways since mitosis is not involved in differentiated cells. [© 1998 Rapid Science Ltd.]

Key words: Cell differentiation, docetaxel, low concentrations, paclitaxel, sequential combination, vinorelbine.

Introduction

Antitubulin agents, which disrupt or stabilize the formation of microtubules (MT), are among the most effective anticancer drugs and have contributed to the cure of neoplasms.[1] These agents target MT and interfere with mitotic spindle function. However, the sites at which these agents act on tubulin and their mechanisms of action at usual concentrations are distinct. Vinca alkaloids depolymerize MT, whereas taxoids stabilize polymerized tubulin into non-functional MT bundles.[2-4] At low doses, these agents have similar kinetics of suppression of MT dynamics, which is especially important at the transition from metaphase to anaphase.[5] Taxoids, vinca alkaloids or colchicine induce apoptosis at the relatively high concentrations causing modifications of the MT network and mitotic block.[6-8] In addition to their specific action on the mitotic spindle, these agents have increased the levels of transcriptionally active p53[9] and inhibited several other genes associated with cell growth such as c-*myc*.[10] Taxol may mediate apoptosis via induction of $p34^{cdc2}$ kinase[11] or the phosphorylation of Bcl-2 protein.[12] Indeed, the balance between antiapoptotic proteins (Bcl-2, Bcl-x_L, etc.) and proapoptotic proteins (Bax, Bak, etc.) regulates apoptosis,[13] their expression varies according to the cell lines and their differentiation state.[14,15] The taxol stabilization of MT or the depolymerization process induced by vinca alkaloids or colchicinoids may also modify the association of some kinases or G-proteins with MT, thereby altering intracellular signaling in response to a variety of stimuli.[16,17]

Vinorelbine ditartrate [Navelbine® (NVB)], a hemisynthetic vinca alkaloid, and the taxoids paclitaxel [Taxol® (TAX)] and docetaxel [Taxotere® (TXT)] have demonstrated clinical activity as single agents against ovarian cancer, breast cancer and non-small cell lung carcinoma.[18,19] However, the best therapeutic results in cancer chemotherapy are usually achieved with drug combinations. Indeed, clinical trials of combinations of paclitaxel

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Correspondence to D Braguer

with other anticancer drugs in a variety of protocols have been initiated.[20,21] The vinca alcaloid and taxoid combinations are currently being tested both *in vitro* and *in vivo*. Their *in vitro* cytotoxic activity against human cell lines largely varies according to duration, drug concentrations and the schedules of tested combinations.[22–25] Preclinical studies on murine models have shown that the taxoid/NVB combination is synergistic, improving survival without enhancing toxic effects.[19,26] The first clinical studies[27–29] of TAX or TXT with NVB suggest that further investigations should be performed in order to define the optimal sequencing and the dose-limiting toxicity of the two drugs. Indeed, the doses tested were the maximun tolerated in humans, i.e. doses inducing mitotic block in cell lines.

We therefore thought that it would be of interest to investigate the sequential combination of NVB and a taxoid over a range of concentrations, from low doses inducing no significant modification of MT network to high doses inducing mitotic block. The study was conducted using the human colon adenocarcinoma cell line HT29-D4. This model does not express the *mdr* gene[30] and it can completely differentiate into an enterocyte-like cell after the substitution of glucose by galactose in the culture medium.[31] Thus, it is particularly suitable to compare the cytotoxicity of antitubulin agents in combination according to the phenotype of cell differentiation. Obviously, the parameters assessed in clinical dose-response studies are tolerance and efficacy, which cannot be transposed to the cytotoxicity measured *in vitro*. However, the activity of antitubulin agents at low concentrations against undifferentiated cells mimics a mechanism of selective cytotoxicity towards malignant cells versus normal cells. Differentiated cells are a good model to demonstrate toxicity on normal cells and/or activity on differentiated cells often present in heterogeneous human adenocarcinoma. Indeed, antitubulin agents at prolonged exposure and high dose may be a potentially interesting clinical application at least in loco-regional therapy against hepatic metastasis of colon tumors including both phenotypes.

We examined sequential combinations of vinorelbine and paclitaxel or docetaxel against undifferentiated and differentiated HT29-D4 cells. The interaction was synergistic only at concentrations not inducing mitotic block. For the two phenotypes, cell death occurred by apoptosis even though apoptotic signaling pathways differ markedly with the state of differentiation.

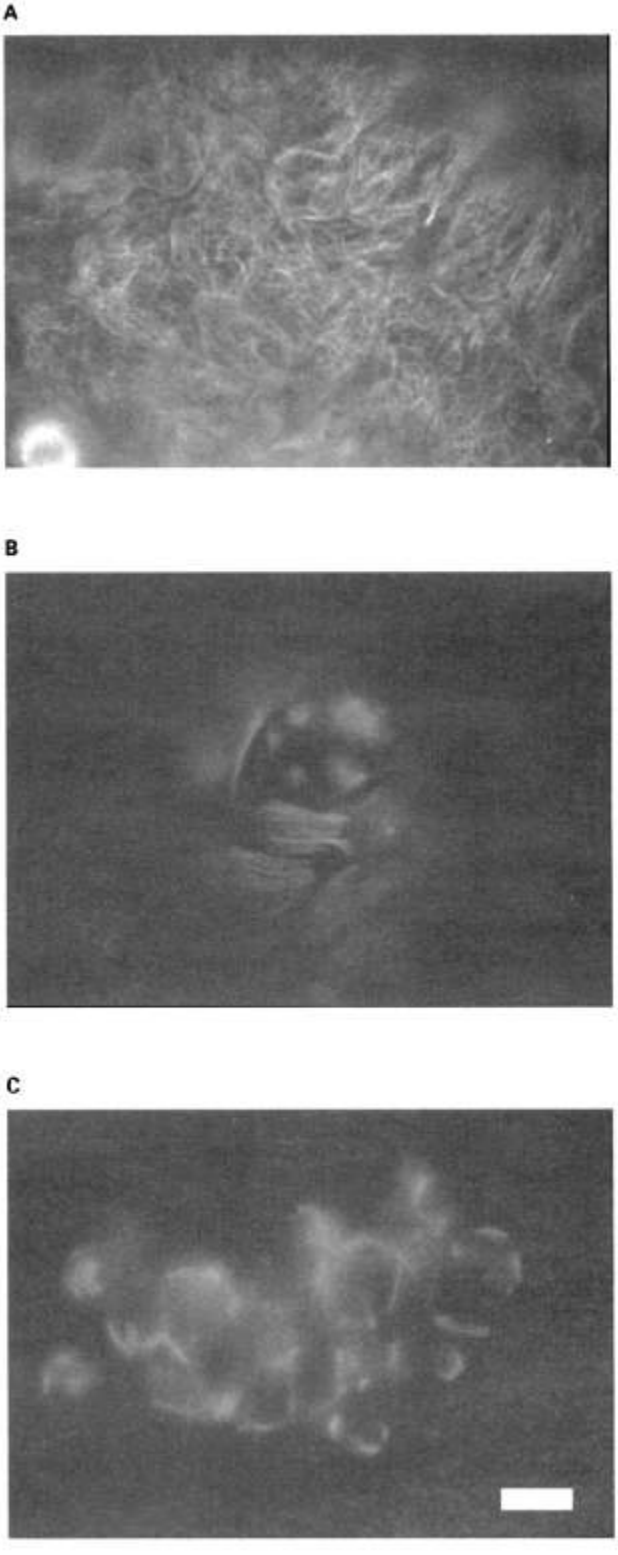

Figure 1. Indirect immunofluorescence staining of α-tubulin in undifferentiated cells. (A) Control cells, (B) cells treated with 25 nM TAX for 24 h showing multiple mitotic asters and bundles and (C) cells treated with 10 μM NVB for 24 h showing paracrystals of tubulin. Bar=10 μm.

Materials and methods

Drugs

Paclitaxel was obtained from Sigma (St Louis, MO). Docetaxel was a gift from Rhône Poulenc Rorer (Paris, France). Stock solutions (0.01 M) of drugs were made in dimethylsulfoxide (DMSO) and were stored at −20°C. The working solutions were diluted in culture medium; the highest concentration of DMSO used was 0.2%. Vinorelbine ditartrate was obtained from Pierre Fabre Oncologie (Paris, France). Stock aqueous solution (1.3×10^{-2} M) was stored at 4°C.

Cell culture and differentiation

Because of the cellular heterogeneity of the HT29 cell line, the HT29-D4 clone was isolated by limited dilution.[31] Undifferentiated HT29-D4 cells were routinely grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Biowhittaker, Verviers, Belgium) containing 25 mM glucose, 10% fetal bovine serum (FBS) (Biowhittaker), 2 mM glutamine and antibiotics (standard medium). Doubling time was 20±2 h. For induction of cell differentiation, standard medium was replaced by DMEM without glucose, supplemented with 5 mM galactose and 10% dialyzed FBS (galactose

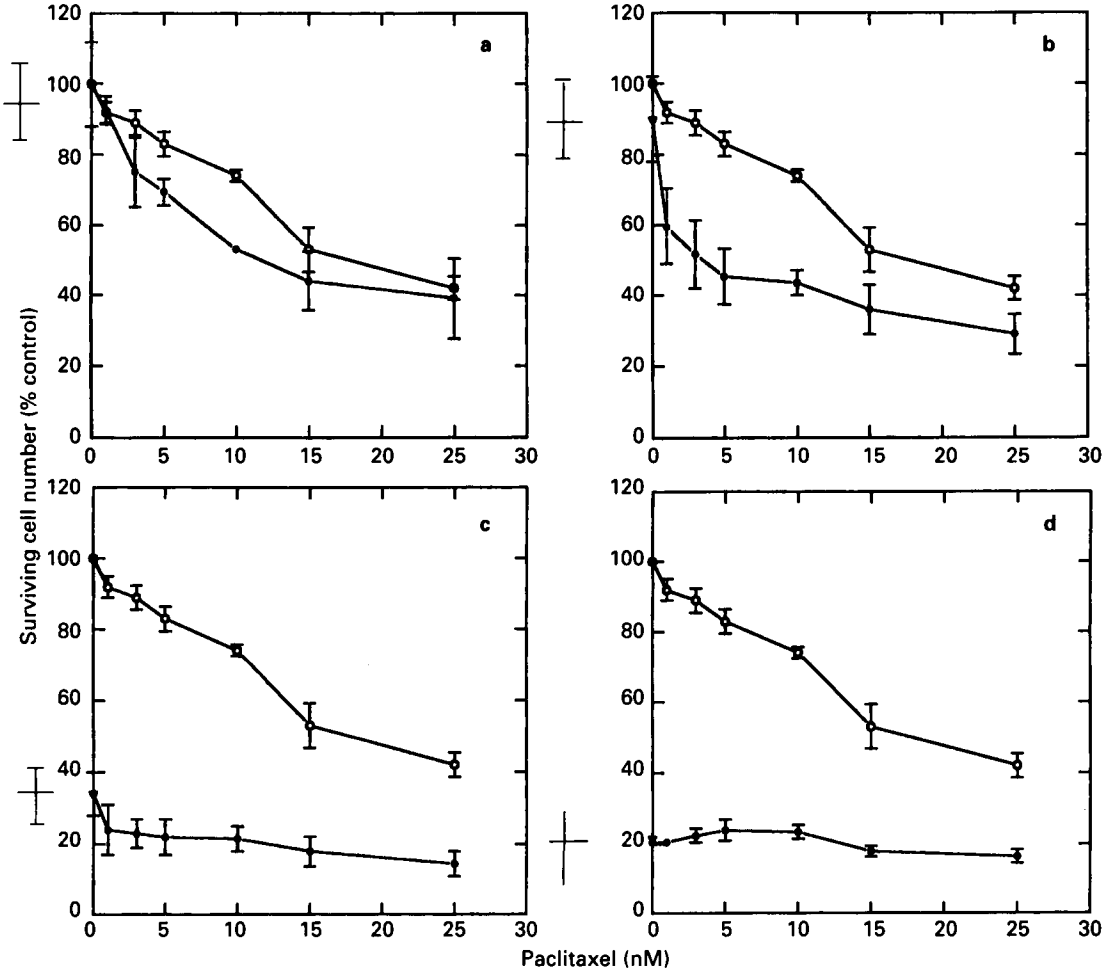

Figure 2. Dose–response curve of undifferentiated cells treated by the sequential combination NVB/TAX. Growth inhibition of the sequential combination (●): (a) 1 nM NVB/TAX (1–25 nM), (b) 2 nM NVB/TAX (1–25 nM), (c) 5 nM NVB/TAX (1–25 nM) and (d) 15 nM NVB/TAX (1–25 nM). Growth inhibition of TAX alone (○) is shown on each curve. The value of NVB alone is on the left of each graph. Each point represents the mean of three separate experiments using triplicate cultures (±SD).

medium). Differentiated HT29-D4 cells were limited to two subcultures.

Drug incubation

Exponentially dividing undifferentiated cells were plated in 96-well tissue culture plates (1.5×10^4 cells/well) for cytotoxicity assays. Six-well tissue culture plates (4×10^5 cells/well) were used for all other experiments. Twenty-four hours later, cells were treated according to the desired sequential schedule: 24 h of treatment with the first compound at various concentrations diluted in the standard culture medium and then 24 h with the second compound alone in the same conditions. Finally, after drug treatment, drug-free medium was added for 24 h.

Cell monolayers of differentiated cell cultures in six-well tissue culture plates (2×10^6 cells/well) were incubated with the TAX/NVB combination (1 μM TAX for 3 days or 0.75 nM TAX for 9 days then with 1 μM NVB for 3 days) or with the NVB/TAX combination (10 nM NVB or 0.75 nM NVB for 9 days then with 1 μM TAX for 3 days). The galactose medium containing the drug was changed daily. Alternatively, paclitaxel was replaced by docetaxel at the same concentrations.

Cytotoxicity assay

For undifferentiated cells, cytotoxicity was assessed by the tetrazolium assay (MTT) or exclusion with Trypan blue (Sigma).[32,33] Before drug exposure, we

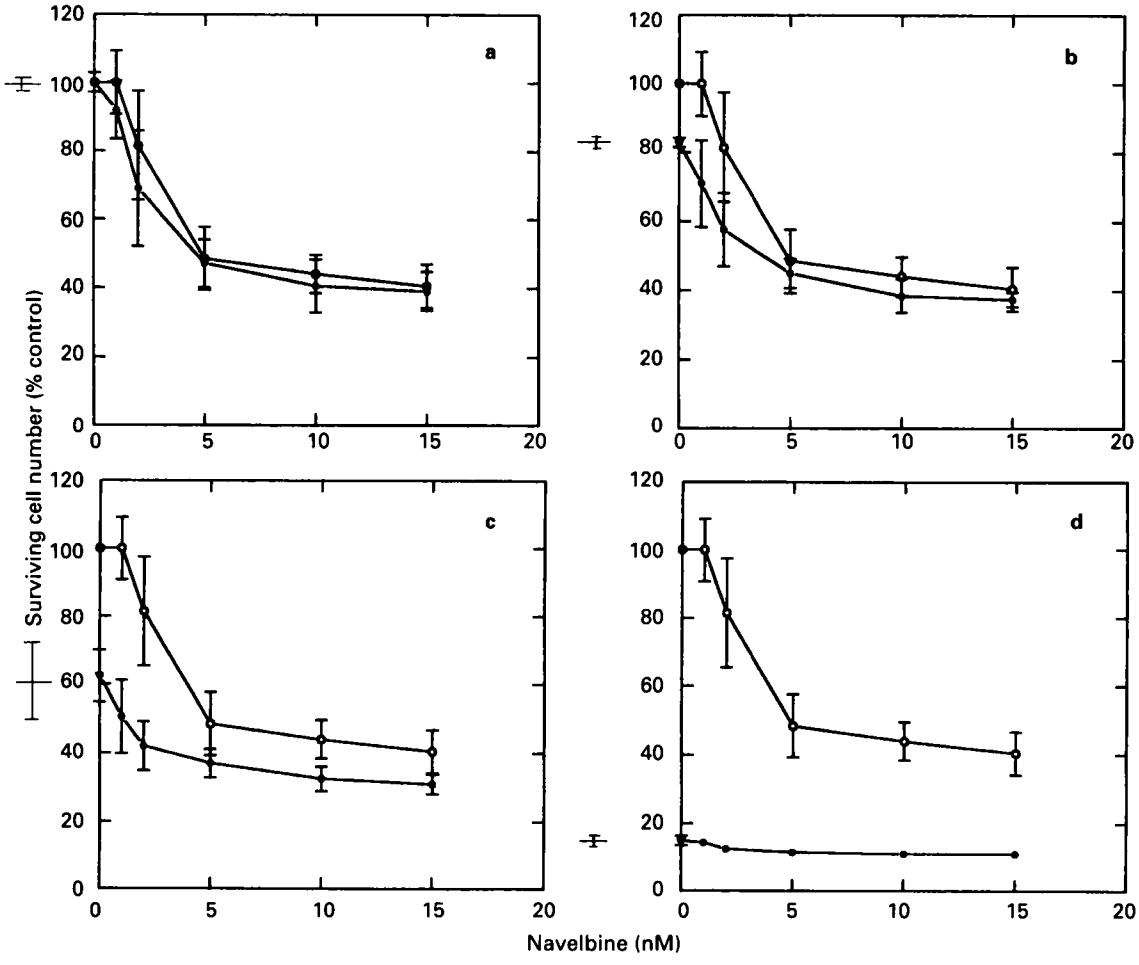

Figure 3. Dose–response curve of undifferentiated cells treated by the sequential combination TAX/NVB. Growth inhibition of the sequential combination (●): (a) 1 nM TAX/NVB (1–15 nM), (b) 3 nM TAX/NVB (1–15 nM), (c) 5 nM TAX/NVB (1–15 nM) and (d) 25 nM TAX/NVB (1–15 nM). Growth inhibition of NVB alone (○) is shown on each curve. The value of TAX alone is on the left of each graph. Each point represents the mean of three separate experiments using triplicate cultures (±SD).

established a linear relation between the MTT assay and cell number. Three independent experiments were performed using quadruplicate wells per concentration.

The IC_{50} was defined as the concentration of drug that produced 50% cell growth inhibition relative to untreated controls. The inhibition caused by the combinations was compared with that caused by each single agent in the same experimental conditions. Inhibition of cell proliferation was considered synergystic if the surviving fraction of cells was less than the product of the surviving fractions obtained by the individual agents at the designated concentration.[34]

Table 1. Analysis of interaction of the combination inhibiting undifferentiated HT29-D4 cell proliferation

Combination of drugs	Observed surviving fraction/predicted surviving fraction	Combination of drugs	Observed surviving fraction/predicted surviving fraction
1 nM NVB + 0 nM TAX	1	1 nM TAX + 0 nM NVB	1
1	1.00 ± 0.09	1	0.92 ± 0.08
3	0.84 ± 0.13	2	0.88 ± 0.04
5	0.83 ± 0.04	5	0.97 ± 0.04
10	0.71 ± 0.07	10	0.91 ± 0.05
15	0.83 ± 0.07	15	0.96 ± 0.01
25	0.92 ± 0.07		
2 nM NVB + 0 nM TAX	1	3 nM TAX + 0 nM NVB	1
1	0.71 ± 0.09	1	0.85 ± 0.14
3	0.64 ± 0.04	2	0.88 ± 0.02
5	0.60 ± 0.04	5	1.10 ± 0.10
10	0.65 ± 0.06	10	1.05 ± 0.04
15	0.75 ± 0.07	15	1.10 ± 0.10
25	0.90 ± 0.10		
5 nM NVB + 0 nM TAX	1	5 nM TAX + 0 nM NVB	1
1	0.76 ± 0.16	1	0.81 ± 0.03
3	0.76 ± 0.04	2	0.90 ± 0.11
5	0.78 ± 0.05	5	1.20 ± 0.20
10	0.85 ± 0.07	10	1.20 ± 0.14
15	0.92 ± 0.04	15	1.20 ± 0.20
25	1.01 ± 0.09		
10 nM NVB + 0 nM TAX	1	10 nM TAX + 0 nM NVB	1
1	1.03 ± 0.05	1	1.02 ± 0.08
3	1.13 ± 0.20	2	1.02 ± 0.13
5	1.25 ± 0.21	5	1.60 ± 0.06
10	1.42 ± 0.04	10	1.70 ± 0.05
15	1.56 ± 0.16	15	1.80 ± 0.01
25	1.78 ± 0.01		
15 nM NVB + 0 nM TAX	1	15 nM TAX + 0 nM NVB	1
1	1.14 ± 0.20	1	0.95 ± 0.07
3	1.25 ± 0.11	2	0.01 ± 0.20
5	1.42 ± 0.09	5	1.40 ± 0.27
10	1.60 ± 0.21	10	1.50 ± 0.19
15	2.49 ± 0.70	15	1.60 ± 0.25
25	2.92 ± 0.90		
		25 nM TAX + 0 nM NVB	1
		1	0.96 ± 0.04
		2	1.02 ± 0.16
		5	1.60 ± 0.21
		10	1.60 ± 0.05
		15	1.80 ± 0.11

The predicted surviving fraction for the combination is the product of multiplying the surviving fraction obtained with the first agent at the designated concentration by the surviving fraction obtained with the second agent at the designated concentration. A value of 1 indicates additivity, values below 1 synergy and values above 1 antagonism. Each point shows the mean of three experiments (± SD)

Microscopy

Immunofluorescence analysis of the MT network and DAPI (Sigma) staining were performed as previously described.[35,36] Apoptotic cells were detected by the ApopTag *in situ* fluorescein assay according to the manufacturer's instructions (Oncor, Gaithersburg, MD).

Cell cycle analysis

Cells were harvested, fixed in cold methanol, incubated with RNase (Type I-1; Sigma) and before analysis were stained with propidium iodide (Sigma). DNA content was measured by flow cytometry (FACScan, Becton Dickinson) as previously described.[4]

Results

Undifferentiated HT29-D4 cells

Inhibition of cell proliferation, mitotic block and modification of the MT network. HT29-D4 cells were incubated for one cell cycle (20 h) with TAX, TXT or NVB over a broad range of concentrations (1–50 nM). The resulting IC_{50} values were 25 nM TAX and TXT and 10 nM NVB. These concentrations correspond to those needed to block cells in mitosis. They also induce the classical modifications of the MT network: depolymerization by NVB and production of

Table 2. Change in cell DNA content in undifferentiated HT29-D4 cells treated with the synergistic combinations NVB/TAX or NVB/TXT at low concentration ($G_0/G_1+S+G_2/M = 100\%$)

Drugs	Percentage of cells		
	G_0/G_1	S	G_2/M
Control	73	6	21
5 nM NVB –[a]	69	6	24
–[a] 1 nM TAX	78	4	17
–[a] 3 nM TAX	75	5	20
–[a] 5 nM TAX	75	5	20
–[a] 5 nM TXT	71	9	20
5 nM NVB/1 nM TAX	51	8	41
5 nM NVB/3 nM TAX	42	8	50
5 nM NVB/5 nM TAX	38	8	54
5 nM NVB/5 nM TXT	37	9	54

[a]Medium free of drug for 24 h.

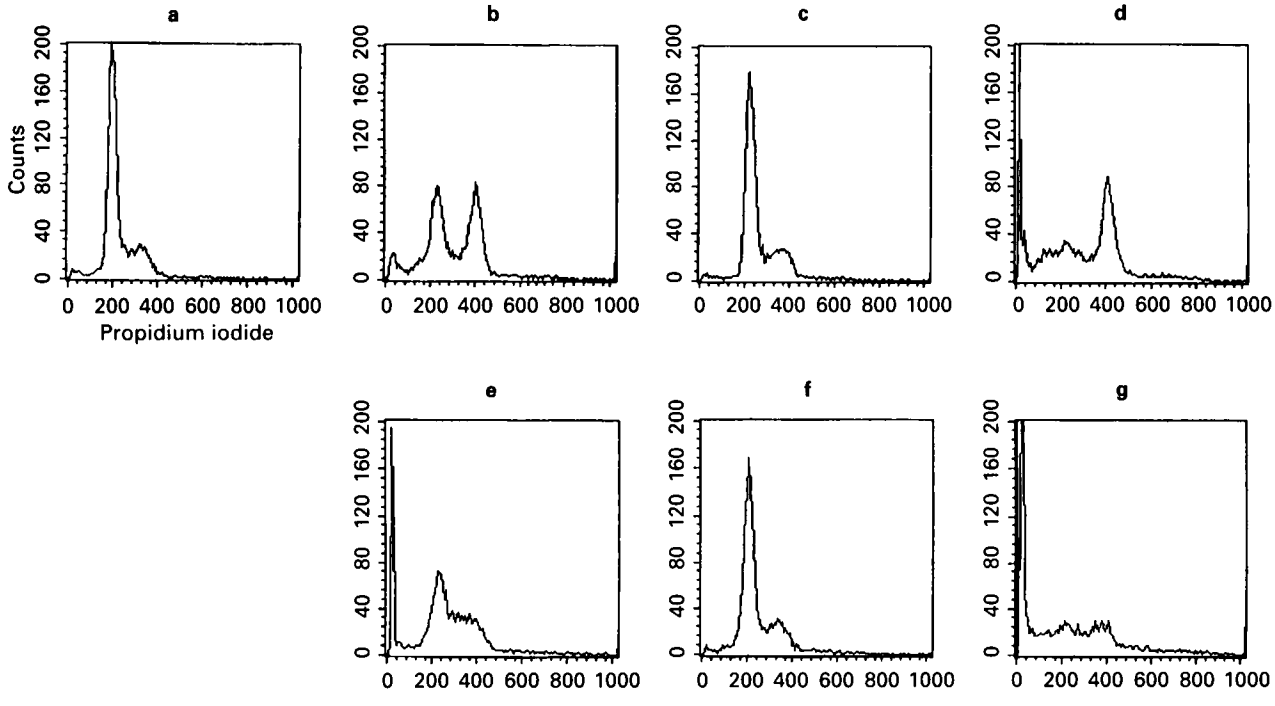

Figure 4. Variation of cell DNA content in undifferentiated HT29-D4 cells. (a) Untreated cells, (b and e) cells treated with 5 nM NVB alone and (c and f) 5 nM TAX alone as control, (d and g) cells treated with the sequential combination NVB/TAX. Panels (b–d) correspond to cell cycle measured at the end of drug treatment and (e–g) to 24 h after drug removal, i.e. when cytotoxicity was quantified.

bundles or pseudo-asters (50/50%) by taxoids. Paracrystals of tubulin were observed in cells treated for 24 h with NVB (1–10 μM) (Figure 1). The lowest concentrations of drugs inducing modification of MT network were 5 nM NVB and 1 nM for TAX and TXT.

Antiproliferative effect of combinations

To determine the optimal sequence of combinations, we compared the effect of 24 h NVB exposure followed by 24 h TAX exposure followed by drug-

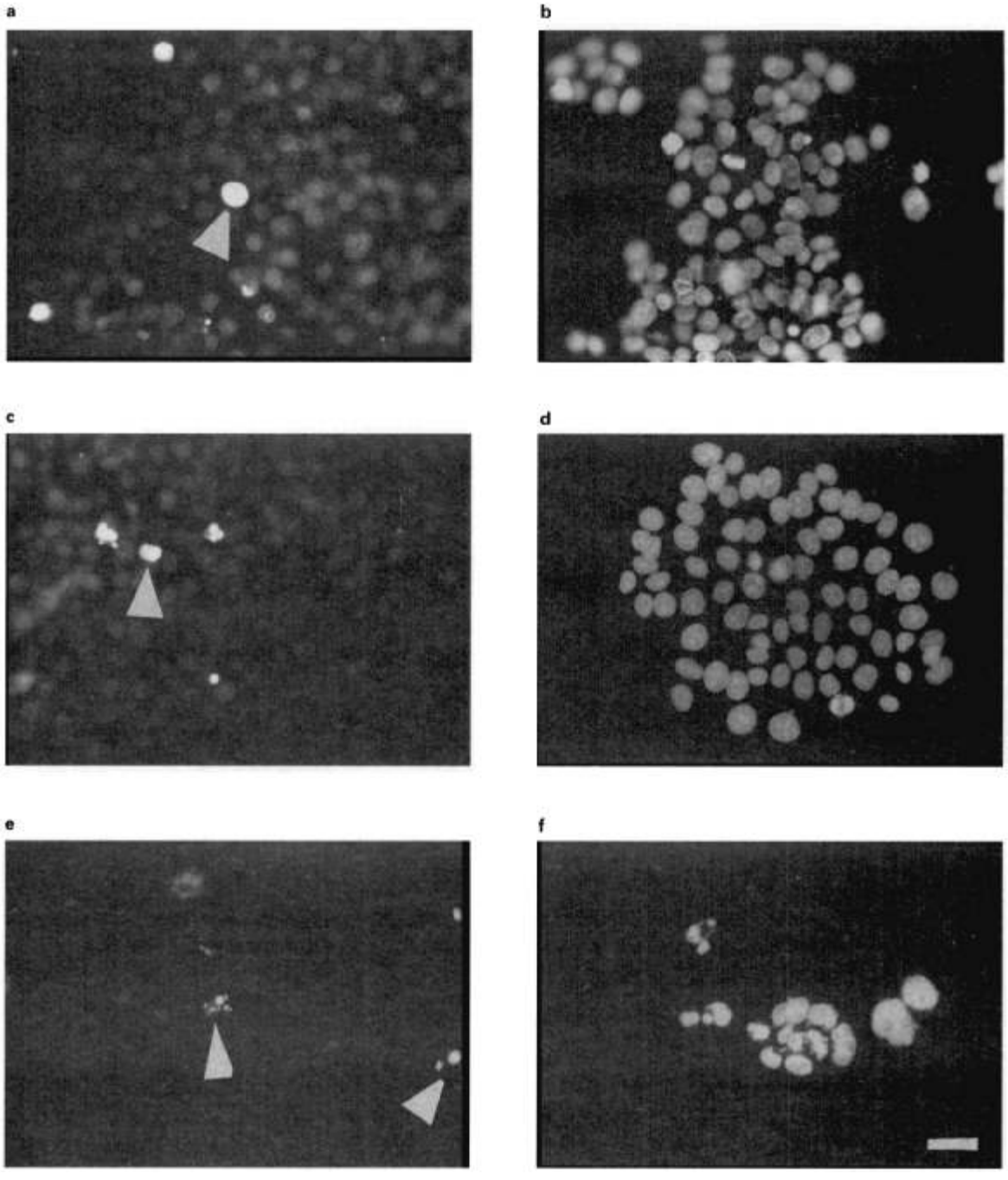

Figure 5. (a, c and e) Apoptosis detected by the ApopTag fluorescein assay and (b, d and f) DAPI staining of undifferentiated cells. (a and b) Cells treated with NVB alone and (c and d) with TAX alone considered as controls of (e and f) the sequential schedule NVB/TAX. The synergistic combination 5 nM NVB/5 nM TAX shows (e) apoptotic cells and (f) multinucleated cells. Arrows indicate apoptotic cells. Bar = 25 μm.

free medium for 24 h (NVB/TAX) with TAX/NVB under the same conditions.

Inhibitions of cell proliferation by the sequential combinations of NVB/TAX and TAX/NVB are shown in Figures 2 and 3. The NVB/TAX sequence appeared more cytotoxic than TAX/NVB [22% cell survival versus 37% for 5 nM TAX and NVB; as shown in Figures 2(c) and 3(c)].

However, data analysis was necessary for confirmation of synergy (Table 1). The ratio values determine the nature of the interaction: a value of 1 indicates additivity, values below 1 synergy and values above 1 antagonism.[34] Treatment with NVB followed by TAX resulted in a substantial synergy in the killing of HT29-D4 cells at doses of each drug lower than those inducing mitotic block: 5 nM for NVB and 20 nM for TAX. Additivity was observed for 25 nM TAX combined with NVB at a dose not inducing mitotic block. Combinations became antagonistic for doses of NVB higher than that inducing mitotic block (10 nM), whatever the dose range of TAX. When the concentrations of each drug were lower than those inducing mitotic block, the sequence TAX/NVB did not produce synergistic effects; only additivity was observed. This interaction became antagonistic when the concentration of either drug reached its IC_{50} value of 10 nM TAX and 8 nM NVB.

The same patterns of synergy, additivity and antagonism were obtained with the sequential combination of NVB followed by TXT when TXT was tested at the same concentrations as TAX (data not shown).

DNA content and apoptosis in the synergistic combination NVB/TAX at low concentration

Cell DNA content. Less than 25% of control cells or cells treated with 5 nM NVB, 5 nM TAX or 5 nM TXT alone were in the G_2/M cell cycle phase (Table 2). On the contrary, treatment with 5 nM NVB/5 nM TAX or 5 nM NVB/5 nM TXT resulted in 54% of cells entering G_2M (Table 2 and Figure 4). This phenomenon correlates directly with TAX concentration (1-5 nM).

Induction of apoptosis. Flow cytometry (Figure 4) and microscopic examination (Figure 5) of cells treated with the sequential combination of NVB followed by a taxoid at low concentration indicated that cell death occurred by apoptosis. At the end of drug incubation, cells were blocked in G_2/M with a 4 *n* amount of DNA (Figure 4d). Twenty-four hours after drug removal, i.e. when cytotoxicity was quantified, 25% of cells treated with 5 nM NVB/5 nM TAX had variable amounts of DNA, generally less than 2 *n* amount (Figure 4g). Only 8% of cells treated with NVB alone showed an amount of DNA less than 2 *n* (Figure 4e) and no significant variation was observed for control cells (Figure 4a) or cells treated with a taxoid alone (Figure 4f). These data were confirmed by the quantification of cells containing nucleosome-size DNA fragments stained with fluorescein (Table 3 and Figure 5). Moreover, DAPI staining indicated that 5 nM NVB/5 nM TAX induced multinucleated cells, whereas cells treated with each compound alone did not (Figure 5).

Table 3. Percentage of apoptotic undifferentiated HT29-D4 cells assessed by the ApopTag fluorescein assay with the sequential schedule 5 nM NVB/5 nM TAX

Drugs	Percentage of apoptotic cells
Control	1
5 nM NVB $-^a$	8±2
$-^a$ 5 nM TAX	2±1
5 nM NVB/5 nM TAX	25±3

Each point represents the mean of two separate experiments (±SD).
[a]Medium free of drug for 24 h.

Table 4. Dose and time effects of TAX alone and NVB alone on differentiated HT29-D4 cell cytotoxicity (viable cells were numerated as described in Materials and methods)

TAX (μM)	Surviving cell number (percent control)				NVB (μM)	Surviving cell number (percent control)		
	3[a]	4[a]	5[a]	6[a]		2[a]	3[a]	6[a]
1	89	80	65	40	1	68	65	45
30	70	67	66	–	30	53	46	–
40	60	40	37	–	50	56	42	–
50	57	39	19	–	75	–	21	–
100	38	–	–	–	100	27	16	–

[a]Days of treatment.

Differentiated HT29-D4 cells

Cytotoxicity of taxoids or NVB alone. No cytotoxicity was observed in differentiated cells treated at doses and exposure times similar to those used on undifferentiated cells. To induce cytotoxicity, we increased the duration of treatment and the drug concentrations. TAX and NVB (100 μM for 3 days) induced, respectively, 38 and 16% of cell survival in differentiated cells (Table 4). The IC_{50} values after 3 days of treatment were 78 and 12 μM for TAX and NVB, respectively. Cytotoxicity was time dependent (Table 4). Indeed, cell survival was 65% for 1 μM TAX for 5 days or 1 μM NVB for 3 days.

No modification in the cell cycle distribution was observed and no pseudo-aster was visualized by immunofluorescence analysis, whatever the dose and time (50 μM NVB for 3 days or 1-50 μM TAX for 3-5 days) (data not shown).

Cytotoxicity of drug combinations. The sequential TAX/NVB and NVB/TAX combinations were tested. The former was the most cytotoxic. However, synergy was observed only when TAX or TXT, at very low dose (0.75 nM) and long-term exposure, was combined with 1 μM NVB (Table 5).

Cell DNA content and apoptosis. No mitotic block was observed whatever the tested combinations (data not shown). Cells treated with the TAX/NVB combinations induced a 20% apoptotic rate, whereas each agent alone induced less than 6% apoptosis (Table 5 and Figure 6).

Discussion

Undifferentiated HT29-D4 cells

The two sequential combinations taxoid/vinca alkaloid and vinca alkaloid/taxoid were tested at various concentrations for their cytotoxic effects on undifferentiated HT29-D4 cells. The concentrations ranged from low doses inducing no modification of MT network to high doses corresponding to the usual clinically relevant concentrations that block mitosis. The combination NVB/TAX was more efficient than TAX/NVB since synergism was clearly evidenced at the lowest concentrations of NVB (1-5 nM) and TAX or TXT (1-15 nM).

The nature of the interaction was dose-dependent: synergism for low doses of both compounds, then additivity and finally antagonism when one of the compounds was at the concentration inducing mitotic block. The same pattern was also obtained with the NVB/TXT combination. Our findings confirm the higher activity of NVB given first,[24] and the dose-dependent activity of TAX and NVB described against human melanoma cell lines.[25] These authors observed a synergistic drug interaction at low concentration, then additivity with higher concentrations for 1 h exposure with the simultaneous combination.

Mitosis is involved, at least partially, in the action of the combination at the lowest concentrations. Indeed, the number of cells in G_2/M is significantly increased by the NVB/TAX combination (54% in G_2/M), whereas treatment with TAX or NVB alone induced no modification, thus supporting a synergistic relation-

Table 5. Effects of sequential combinations on differential HT29-D4 cells: cytotoxicty and analysis of interaction [each point shows the mean of three experiments (±SD)]

Sequential combination	Percent of cell surviving	Observed surviving fraction/ predicted surviving fraction
1 μM TXT 3d/1 μM NVB 3d	52±2	1.50±0.09
1 μM TAX 3d/1 μM NVB 3d	44±3	1.18±0.03
–[a] 1 μM NVB 3d	62±5	–
1 μM TAX 3d –[a]	55±5	–
0.75 nM TXT 9d/1 μM NVB 3d	38±8	0.60±0.05
0.75 nM TAX 9d/1 μM NVB 3d	48±5	0.72±0.01
–[a] 1 μM NVB 3d	62±5	–
0.75 nM TAX 9d –[a]	96±0	–
0.75 nM NVB 9d/1 μM TAX 3d	96±2	1.05±0.03
0.75 nM NVB 9d –[a]	95±6	–
–[a] 1 μM TAX 3d	93±7	–
10 nM NVB 9d/1 μM TAX 3d	61±3	1.03±0.05
10 nM NVB 9d –[a]	64±2	–

[a]Medium free of drug for similar time. d = days.

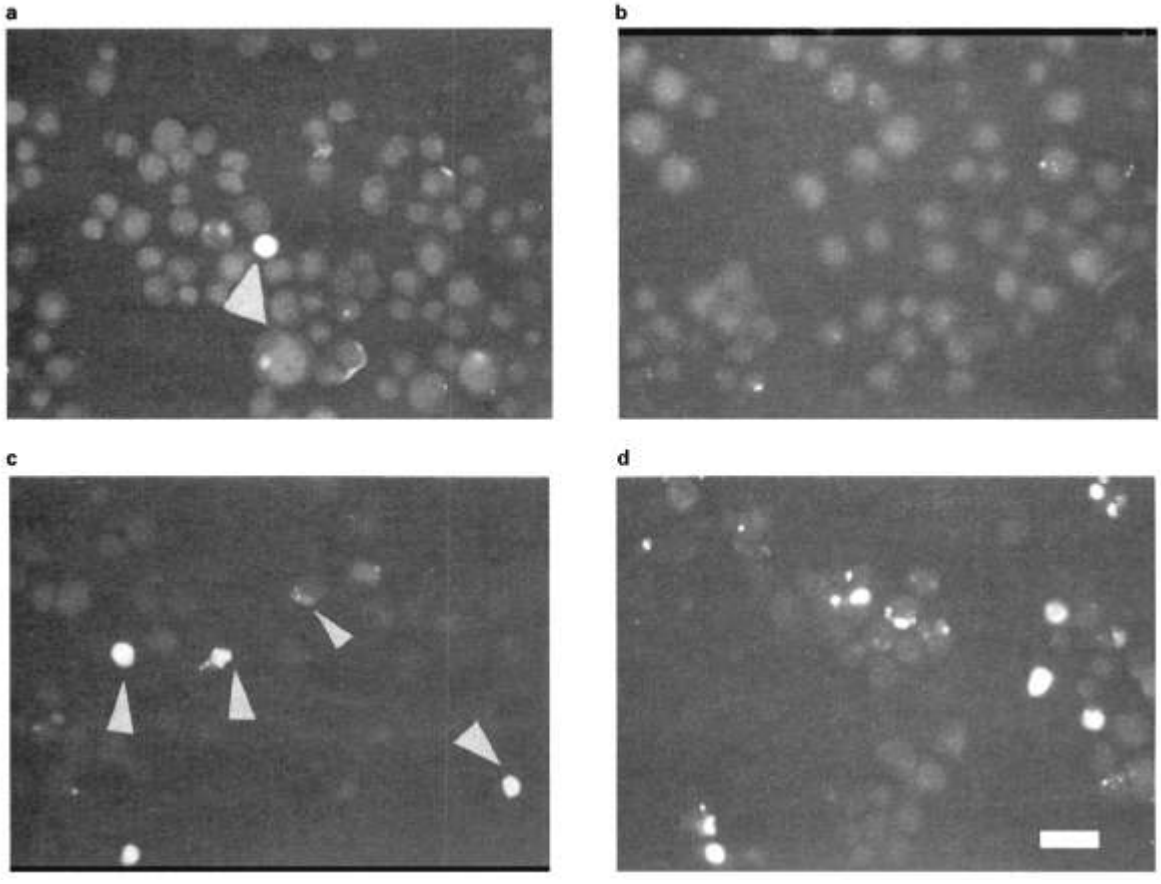

Figure 6. Apoptosis of the differentiated cells: (a) control cells, (b) cells treated with 0.75 nM TAX alone for 9 days and (c) 1 μM NVB alone for 3 days as controls or (d) 0.75 nM TAX for 9 days followed by 1 μM NVB for 3 days. Arrows indicate apoptotic cells. Bar = 25 μm.

ship. It was hypothezised that at low concentrations depolymerizing agents produce cytotoxicity by interfering with the dynamics of spindle MT as part of spindle function, whereas taxoids produce cytotoxicity by interfering with spindle formation.[38] Both mechanisms of mitotic spindle modification with MT agents at very low concentrations may be involved. We previously obtained similar results (i.e. an increase in mitotic block) with simultaneous combinations of taxoid/colchicinoid at low doses on KB 3-1 cells and J82 cells.[33]

The synergistic interaction with the NVB/TAX combination at low concentrations against undifferentiated cells may be compared with apoptosis induced with low taxol concentration (10 nM) in HeLa cells.[38] Indeed, cells progress in mitosis at the end of the treatment, then an abnormal exit from mitosis occurs without cytokinesis. Further cell proliferation is inhibited and apoptosis occurs. Such a mechanism appears not to exist in cells treated with each compound alone because their concentration was too low to induce a partial mitotic block, which would appear to be determinant in the triggering of apoptosis. For higher concentrations of drugs, additivity corresponds to the cumulative effect of each agent on variation of cell cycle. When the concentration of one drug was increased sufficiently to induce total mitotic block, we did not observe more cells blocked in G_2/M and did not detect more cytotoxicity after adding a second compound. This therefore accounts for the antagonism of the interaction.

Differentiated HT29-D4 cells

HT29-D4 cells are completely differentiated into an enterocyte-like cell characterized by polarization and apical brush border forming a monolayer exclusively composed of differentiated cells with tight junctions and lack of proliferation.[31] Differentiated cells are much less sensitive to antitubulin agents than undifferentiated cells: they require dramatically higher

concentrations (IC_{50} about 1000 times) and longer exposure time. As expected, measurement of cell cycle confirmed that the cell killing does not occur by mitosis.

The first agent in the combination was tested at a very low concentration (0.75 nM NVB or TAX) inducing no modification of the MT network even on undifferentiated cells. When increasing to 10 nM NVB, the sequence NVB/TAX did not show cytotoxicity greater than that of single agents. The percentage of cell death was similar for the two schedules TAX/NVB. However, the combination including a taxoid at 0.75 nM appears synergistic since taxoid alone at this concentration does not induce any cytotoxicity. Cell death occurs by apoptosis independently of a mitotic process, as confirmed by the absence of mitotic figures and the absence of change in cell cycle distribution.

Comparison between undifferentiated and differentiated cells

Our results demonstrate two major differences depending on cell phenotype: (i) the differentiated cells were much less sensitive to antitubulin agents than the undifferentiated cells and (ii) the apoptotic pathways were different.

Differentiated HT29-D4 cells are quiescent and polarized with a stabilized MT network.[39] Stable MT often contain post-translationally modified α-tubulin (acetylated and detyrosinated)[40] and overexpression of MAPs such as MAP E-115[41] and MAP4.[42] Moreover, altered expression of specific β-tubulin isotypes may vary in dividing or resting cells[43] and in taxol-resistant cells.[44] Some of these modifications would seem to be implicated in our model and could explain their low sensitivity to antitubulin agents (manuscript in preparation).

As for the induction of apoptosis, paclitaxel has been shown to activate Raf-1 and cause phosphorylation of Bcl-2 during the G_2/M phase of the cell cycle; therefore resulting in decreased Bcl-2 binding to the proapoptotic Bax protein and subsequent increase in free bax and apoptosis.[45] However, HT29-D4 cells contain little Bcl-2 protein and high levels of Bcl-x_L protein (data not shown).[46] The latter seems to exert an antiapoptosis effect similar to Bcl-2 after the induction of mitotic arrest by paclitaxel.[47] Such a pathway may be implicated in undifferentiated cycling cells.

Some modifications in apoptotic protein cascade might exist in differentiated cells. The differentiation of neuroblastoma enhances Bcl-2 expression and induces alterations of apoptosis to cytotoxic agents.[15] Expression of Bcl-2 and Bcl-x_L tends to occur in a reciprocal fashion during differenciation.[48] In epithelial intestinal cells and in HT29 cells treated with sodium butyrate, Bak expression is increased comparatively to colon neoplasia and HT29 control cells.[49] These authors suggest that Bak overexpression would render terminally differentiated colonic enterocytes highly susceptible to apoptotic stimuli. p53 is one of the proteins that plays a central role in the response to anticancer agent treatment, but taxol apoptosis seems independent of p53[50] and proliferating HT29 cells have mutant p53.[46] Moreover, the differentiated phenotype in HT29 cells has been associated with a significant decrease in cytoskeleton-associated tyrosine proteine kinase activity and marked activity of alkaline phosphatase that may modify the function of protein cascade in apoptosis.[51]

Finally, another mechanism independent of the apoptotic proteins may be envisaged. Indeed, the sequence 0.75 nM TAX/1 μM NVB is synergistic on differentiated cells, suggesting that long-term treatment with a taxoid at very low concentrations may slightly decrease the status of differentiation. Apoptosis could result from an uncoordonated attempt of non-dividing cells to re-enter the cell cycle as described in differentiated PC12 cells.[52]

Conclusion

At present, human colon tumors are notoriously chemoresistant to most therapeutic agents, particularly because of the frequent expression of the MDR phenotype. This explains the non-therapeutic use of MT agents in these diseases.[53] It can be expected that new derivatives of taxoids independent of MDR could be prescribed for colon cancers. Within this context, the HT29-D4 model is particularly suitable.

This study demonstrates that the combination of a MT depolymerizing agent and a MT stabilizing agent induces cytotoxicity on HT29-D4 cells at concentrations determined by the state of differentiation. The nature of the interaction varies according to the concentrations of drugs; synergism is observed at the lower concentrations for the two states. Cytotoxicity occurred by apoptosis. Interestingly, the signal pathways for apoptosis are markedly different since the mitotic process is involved only on undifferentiated cells. Thus, antitubulin agents in combination induce intracellular signaling events which may have a role in mediating apoptosis by a mechanism that may be dependent on a signaling pathway distinct from regulation of mitosis.

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