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Short communication

Sensitivity of different resistant tumour cell lines to the two novel compounds (2Z,4E)-2-methylsulfanyl-5-(1-naphthyl)-4-nitro-2,4-pentadienoate and (1E,3E)-1,4-bis(2-naphthyl)-2,3-dinitro-1,3-butadiene

Maurizio Viale [a,*], Giovanni Petrillo [b], Massimo Maccagno [b], Patrizio Castagnola [c], Cinzia Aiello [a], Cinzia Cordazzo [a], Maria A. Mariggiò [d], Sushilkumar A. Jadhav [b], Lara Bianchi [b], Giuseppe Leto [e], Egon Rizzato [e], Alessandro Poggi [f], Domenico Spinelli [e]

[a] Istituto Nazionale per la Ricerca sul Cancro, S.C. Terapia Immunologica, L.go R. Benzi 10, 16132 Genova, Italy
[b] Dipartimento di Chimica e Chimica Industriale, Università di Genova, Via Dodecaneso 31, 16146 Genova, Italy
[c] Istituto Nazionale per la Ricerca sul Cancro, L.go R. Benzi 10, 16132 Genova, Italy;
[d] Dipartimento di Scienze Biomediche e Oncologia Umana, Università di Bari, Osp. Policlinico, P.za G. Cesare 11, 70124 Bari, Italy
[e] Dipartimento di Chimica Organica "A. Mangini", Università di Bologna, Via San Giacomo 11, 40126 Bologna, Italy
[f] Istituto Nazionale per la Ricerca sul Cancro, S. C. Oncologia Sperimentale D, L.go R. Benzi 10, 16132 Genova, Italy

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ABSTRACT

The inhibition of cell proliferation by methyl (2Z,4E)-2-methylsulfanyl-5-(1-naphthyl)-4-nitro-2,4-pentadienoate (1-Naph-NMCB) and (1E,3E)-1,4-bis(2-naphthyl)-2,3-dinitro-1,3-butadiene (2-Naph-DNB) has been studied in vitro against four cell lines selected for their resistance to doxorubicin, cisplatin, taxol and 5-fluorouracil. In previous experiments both compounds showed good in vitro antiproliferative, cytotoxic and pro-apoptotic activities against cell lines of different histologic origin.

The results of the experiments presented here suggest that 1-Naph-NMCB is able to overcome all of the different mechanisms of resistance showed by the resistant cell lines used for our experiments. On the contrary, when we used the taxol-resistant A549-T12 cell line, characterized by a mechanism of resistance due to a mutation of the target site of taxol on microtubules, it displayed a partial but significant cross-resistance to 2-Naph-DNB. Although the actual mechanism of this cross-resistance has not yet been definitively elucidated, our results from immunostaining of microtubules suggest that it may be linked to the presence of a shared target site for taxol and 2-Naph-DNB on microtubules.

1. Introduction

In an attempt to generate new antitumour compounds with good properties in terms of antiproliferative and pro-apoptotic activity as well as of low toxicity in vivo and ability to overcome the most frequent mechanisms of resistance responsible for the failure of a high number of chemotherapeutic cancer treatments, we synthesized (2Z,4E)-2-methylsulfanyl-5-(1-naphthyl)-4-nitro-2,4-pentadienoate (1-Naph-NMCB, Petrillo et al., 2008) and (1E,3E)-1,4-bis(2-naphthyl)-2,3-dinitro-1,3-butadiene (2-Naph-DNB, Viale et al., 2007). The first compound is basically the result of the application of the "molecular simplification strategy" (Manetti et al., 2000; Crisòstomo et al., 2006) on the lead compound (1E,3E)-1,4-bis(1-naphthyl)-2,3-dinitro-1,3-butadiene (1-Naph-DNB, Viale et al., 2004; Novi et al., 2004; Dell'Erba et al., 2005), whereas the second molecule is a structural isomer of the latter, characterized by a different spatial arrangement (Fig. 1).

When tested in vitro for its inhibition of cell proliferation and apoptotic activity 1-Naph-NMCB showed a significant activity at micromolar concentrations, in particular against the MDA-MB-231 breast cancer cell line, and with a significant general improvement compared to 1-Naph-DNB (Petrillo et al., 2008). In particular, for apoptosis, 1-Naph-NMCB showed an activity that was in some cases better than that observed for 1-Naph-DNB, as evaluated by the morphological analysis of nuclear segmentation, the staining with Annexin V and the analysis in western blot of p53 oncosuppressor protein (Petrillo et al., 2008). Moreover, both the analysis of formation of interstrand cross-links and the analysis of the inhibition of restriction enzyme cutting activity in λ phage DNA treated with the compound (unpublished data) demonstrate the binding of 1-Naph-NMCB to DNA, although lower than that observed for the parent compound 1-Naph-DNB (Petrillo et al., 2008).

In vivo studies allowed the toxicological (determination of lethal and maximal tolerated doses) and pharmacological evaluation of 1-Naph-NMCB. Our findings showed that our compound was characterized by negligible histological toxic effects and an antitumour activity which was in some cases even better than that showed by 1-Naph-

* Corresponding author. Tel.: +39 010 5737320.
E-mail address: maurizio.viale@istge.it (M. Viale).

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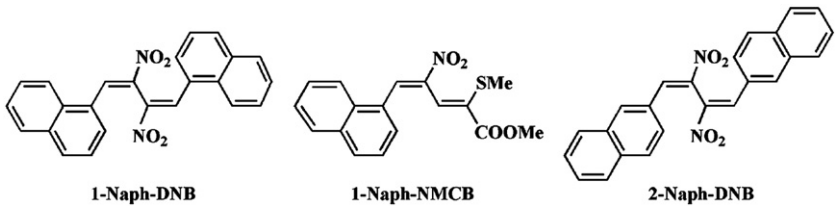


Fig. 1. Molecular structure of 1-Naph-DNB, 1-Naph-NMCB and 2-Naph-DNB.

DNB, and was characterized by differences in tumour selectivity (Petrillo et al., 2007).

2-Naph-DNB showed significant features in terms of inhibition of cell proliferation, with a better activity than 1-Naph-DNB in MDA-MB-231 cells and HGC-27 human gastric cell line, and induction of apoptosis which was linked to the activation of p53 protein and the formation of interstrand cross-links to DNA (Viale et al., 2007). The latter observation was also confirmed by experiments using treated λ phage DNA and restriction enzymes (unpublished data). As for 1-Naph-NMCB, also 2-Naph-DNB was characterized in vivo by low toxic effect at histological level.

All these features prompted us to verify the ability of our molecules to overcome the different mechanisms of resistance showed by some cell lines selected in vitro for their resistance to important anticancer drugs such as doxorubicin, taxol, cisplatin and 5-fluorouracil, also in order to gain further insights into their mechanisms of action.

2. Materials and methods

2.1. Chemicals

The two naphthylnitrobutadienes 2-Naph-DNB and 1-Naph-NMCB were synthesized from 3,4-dinitrothiophene (3,4-DNT) (Viale et al., 2007) and methyl 4-nitrothiophene-2-carboxylate (2-COOMe-4-NT) (Petrillo et al., 2007) as already described (Scheme 1).

While 1-Naph-NMCB differs from the lead compound 1-Naph-DNB (Petrillo et al., 2008) since it contains only one of the two original naphthylnitroethenyl moieties, the isomeric 2-Naph-DNB simply differs from 1-Naph-DNB for a different spatial arrangement of the two naphthyl groups. Such modifications had been devised as initial steps towards the identification of a structure–activity relationship within such a class of potentially-active derivatives.

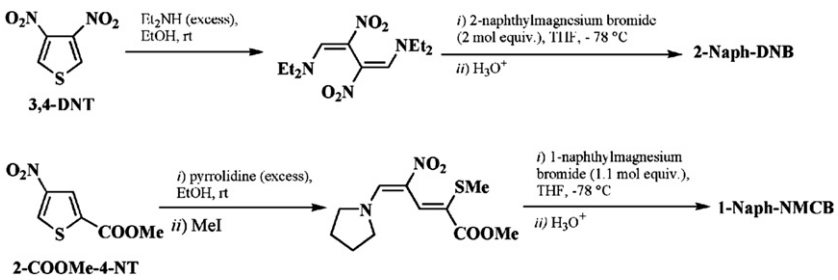
2.2. MTT assay

The resistant cell lines: A2780/DX3 (human ovarian carcinoma, provided by Dr. YM Rustum), A549/T12 (human lung carcinoma, provided by Dr. SB Horwitz), L1210/DDP (murine leukemia, provided by Dr. F Zunino and initially selected at the NCI, Bethesda, Md), HCT-8/FU7dR (human colon cancer, provided by Dr. A Sobrero), and their sensitive counterparts (A2780, A549, L1210 and HCT-8) were plated in opportune densities/well into 96-well microtiter plates for 8 h. 1-Naph-NMCB, 2-Naph-DNB or the other anticancer drugs (doxorubicin, taxol, cisplatin and 5-fluorouracil) were administered in duplicate for a minimum of 5 concentrations (2–5 fold serial dilutions, maximal volume/well 200 μl). Seventy two hours later 50 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO, USA) solution (2 mg/ml in PBS) was added to each well and incubated for 4 h at 37 °C. Microplates were centrifuged at 275 g for 5 min, culture medium carefully aspirated and 100 μl of 100% dimethylsulfoxide added. Complete and homogeneous solubilization of formazan crystals was achieved after 20 min of incubation and vigorous shaking of well contents with a multichannel pipette. The absorbance was measured on a microculture plate reader 400 ATC (SLT Labinstruments, Austria) at 540 nm (Hussain et al., 1993). IC_{50}s were calculated on the basis of the analysis of single dose response curves. Each experiment was repeated 5–12 times to allow the calculation of the mean IC_{50}.

MTT assay was also used to evaluate the exponential growth curves of the cell lines and calculate their doubling time.

2.3. Microtubule immunofluorescence

A549 and A549-T12 cells were cultured in chamber slides and treated with 50 nM taxol, 60, 34 and 17 μM 2-Naph-DNB, 5.6 and 17.3 μM 1-Naph-NMCB and 2 μM cisplatin (as negative control) for 24 h. After incubation cells were washed twice with cold phosphate buffered saline (PBS) and fixed with 3.7% paraformaldehyde in PBS with 2% sucrose (PBS-S) for 5 min at room temperature. Cells were then washed 3 times with cold PBS-S on ice, incubated with methanol for further 5 min at −20 °C and washed again 3 times with cold PBS-S on ice. Non-specific immunoglobuline binding sites were saturated by 30 min incubation with cold 20% goat serum in PBS (GS-PBS) on ice. Cells were then probed with the monoclonal anti-α-tubulin (T 5168, Sigma) diluted 2000 times in GS-PBS for 2 h on ice. Unbound primary antibody was eliminated by 3 washes with cold PBS-S on ice. The Alexa 594 goat anti-mouse IgG1 (Invitrogen, Nelson, UK), at the concentration of 2.5 μg/ml in GS-PBS, was used to reveal the anti-α-tubulin antibody by incubating the cells on ice for 30 min. The samples were washed 3 times with cold PBS-S on ice and the slide was mounted with a coverslip using GelMount (Biomeda Inc., CA). Images were acquired with an Axiovert 200 M microscope (Zeiss).



Scheme 1.

2.4. Analysis of the cell cycle

A549 and A549-T12 cells were studied for the effect of 1-Naph-NMCB, 2-Naph-DNB and taxol on cell cycle phases. To this end cells ($0.75–2\times10^6$) were treated for 24 h with their specific IC_{50}s, harvested, washed twice with cold phosphate buffered saline and fixed in 70% ethyl alcohol at −20 °C overnight. Following fixation, cells were centrifuged, washed with phosphate buffered saline, and incubated at room temperature for 20 min with propidium iodide staining solution (20 mg/ml RNase A in phosphate buffered saline, 50 μg/ml propidium iodide, 0.05% Triton X-100). Cells were then analyzed by flow cytometric analysis of DNA content using a FACSort flow cytometer (BD Biosciences, Mountain. View, CA). The percentages of cell cycle distribution were calculated by the ModFit LT computer program.

2.5. Statistical analysis

The Mann–Whitney test for non-parametric data was used for the statistical analysis (StatView 4.5 software, Abacus Concepts Inc., Burlington, MA, USA). The resistance indexes were calculated as the ratio between the mean IC_{50} of resistant cells and that of corresponding wild-type cells. Only cells with resistance index >2.5 were arbitrarily considered resistant. Lower values were considered too subtle to be pharmacologically relevant, also if statistically significant.

3. Results

3.1. Inhibition of cell proliferation and morphology of microtubules

In human A2780 cells, sensitive (doubling time 24 h) and resistant to doxorubicin (doubling time 27 h), both 1-Naph-NMCB and 2-Naph-DNB were active at the micromolar concentration level. Both molecules were less active than doxorubicin in sensitive A2780 cells, on the other hand their IC_{50}s for resistant and sensitive cells were nearly similar. On the basis of the results showed in Table 1, the resistance indexes for doxorubicin, 1-Naph-NMCB and 2-Naph-DNB were 63.0, 1.8 and 1.2, respectively. No cross-resistance was observed between doxorubicin and the nitrobutadiene compounds (Table 1).

In sensitive (doubling time 11 h) and resistant to cisplatin (doubling time 13 h) L1210 cells both 1-Naph-NMCB and 2-Naph-DNB were similarly active (resistance indexes 0.7 and 1.1, respectively) although 1-Naph-NMCB was more effective than 2-Naph-DNB in resistant L1210/DDP cells (resistance index for cisplatin 19.7) (1.79±0.33 vs 5.35±1.53 μM, $P=0.0009$). Both 1-Naph-NMCB and 2-Naph-DNB were less active than cisplatin in sensitive L1210 cells (0.39±0.17 vs 2.58±0.35 μM, $P=0.0004$; vs 5.00±0.41 μM, $P=0.0034$, respectively) (Table 1).

Considering HCT-8/FU7dR cells resistant to 5-fluorouracil (doubling time 36 h, resistance index 9.9) and their sensitive (doubling time 45 h) counterpart, both 1-Naph-NMCB and 2-Naph-DNB showed a significantly better activity than 5-fluorouracil (HCT-8: 20.00±6.00 vs 12.66±2.43 μM, $P=0.0046$; vs 11.8±2.87 μM, $P=0.0025$, respectively; HCT-8/FU7dR: 197.6±54.4 vs 16.85±4.07 μM, $P=0.0006$; vs 22.38±0.98 μM, $P=0.0027$, respectively) (Table 1). The resistance indexes of HCT-8/FU7dR for 1-Naph-NMCB and 2-Naph-DNB were 1.3 and 1.9, respectively.

Also in A549 cells sensitive (doubling time 25 h) and resistant to taxol (doubling time 30 h) our two nitrobutadiene compounds were significantly less active than taxol (A549: 0.021±0.005 vs 5.95±1.67 μM, $P<0.0001$, vs 13.37±3.44 μM, $P=0.0022$, for taxol, 1-Naph-NMCB and 2-Naph-DNB, respectively; A549-T12: 0.219±0.058 vs 15.08±3.52 μM, $P=0.0039$, vs 67.45±14.51 μM, $P=0.015$, for taxol, 1-Naph-NMCB and 2-Naph-DNB, respectively). Moreover, while for 1-Naph-NMCB the observed resistance index was 2.5, the value raised to 5.0 for 2-Naph-DNB.

As we arbitrarily considered resistant those cells having a resistance index>2.5, we performed some experiments in order to identify the mechanism involved in the resistance phenomenon showed by A549-T12 cells treated with 2-Naph-DNB (resistance index 5.0).

The presence of cross-resistance in these cells suggested that microtubules might be one of the possible targets (if not the main one) of our nitrobutadiene derivative. Thus, by immunofluorescence microscopy, we verified the ability of 2-Naph-DNB to alter the organization of the microtubule network supposed to be one of the target of its action. As shown in Fig. 2D,E,F the exposure of A549 to 2-Naph-DNB alters the normal organization of microtubules through the formation of bundles, more evident at the higher dose used (Fig. 2D). Taxol, a well-known microtubule-stabilizing agent, formed typical bundles (Fig. 2B). As expected in case of a partial or complete sharing of the target site on microtubules, similar doses of both taxol and 2-Naph-DNB applied to taxol-resistant A549-T12 cells displayed negligible or absent bundling activity (Fig. 2C, I). As expected, when treated with 1-Naph-NMCB A549 cells showed very rare or no formation of typical bundles (Fig. 2G,H).

3.2. Cell cycle

The analysis of variation of cell cycle phases on sensitive A549 and resistant A549-T12 cells showed that both 1-Naph-NMCB and 2-Naph-DNB at their IC_{50}s were able to cause a partial but significant accumulation of cells in the G2/M phase of the cell cycle, lower than that caused by the administration of an equitoxic concentration of

Table 1
Inhibition of cell proliferation of 2-Naph-DNB and 1-Naph-NMCD on cells lines sensitive and resistant to cisplatin, doxorubicin, 5-fluorouracil and taxol

Cell lines	Drugs and new nitrobutadiene derivatives					
	Cisplatin	Doxorubicin	5-Fluorouracil	Taxol	2-Naph-DNB	1-Naph-NMCB
A2780	–	0.04±0.02[a] (11)	–	–	3.91±1.16 (12)	2.11±0.74 (12)
A2780/DX3	–	2.51±0.33 (11)	–	–	6.89±1.53 (6)	2.52±0.44 (10)
L1210	0.39±0.17 (8)	–	–	–	5.00±0.41 (5)	2.58±0.35 (10)
L1210/DDP	7.68±2.02 (10)	–	–	–	5.35±1.53 (9)	1.79±0.33 (9)
HCT-8	–	–	20.00±6.00 (10)	–	11.80±2.87 (10)	12.66±2.43 (12)
HCT-8/FU7dR	–	–	197.60±54.4 (7)	–	22.38±0.98 (6)	16.85±4.07 (10)
A549	–	–	–	0.021±0.005 (12)	13.37±3.44 (9)	5.95±1.67 8 (10)
A549-T12	–	–	–	0.219±0.058 (6)	67.45±14.51 (9)	15.08±3.52 (6)

The specific resistance indexes of L1210/DDP, A2780/DX3, HCT8/5FU and A549-T12 are: 19.7, 63.0, 9.9 and 10.4, respectively.
The resistance indexes of L1210/DDP, A2780/DX3, HCT8/5FU and A549-T12 treated with Naph-DNB-2 are: 1.1, 1.8, 1.9 and 5.0, respectively, and treated with 1-Naph-NMCD are: 0.7, 1.2, 1.3 and 2.5, respectively.
[a] Values represent the mean IC_{50}±S.D. (μM) of experiments whose number is reported in parenthesis.

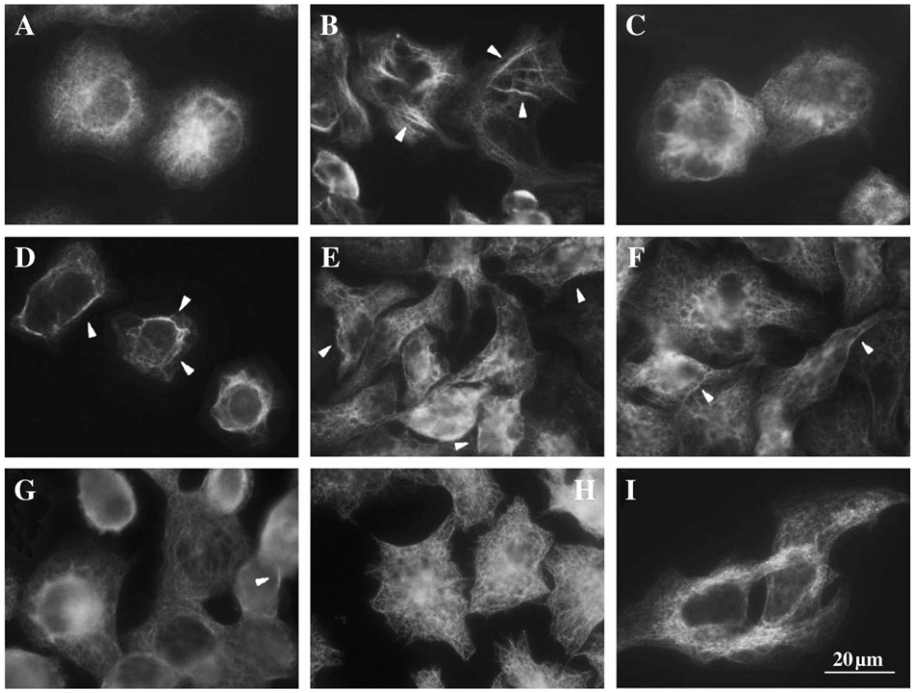


Fig. 2. Effect of 2-Naph-DNB and taxol on the cellular organization of microtubule network in sensitive and resistant to taxol A549 and A549-T12 cells. A, untreated sensitive A549 cells. B, A549 cells treated with 50 nM taxol. Note the rearrangement of microtubules reorganized in typical bundles (arrowheads). C, resistant A549-T12 cells treated with 50 nM taxol. Note the absence of formation of bundles. D, E, F, sensitive A549 cells treated with 60, 34 and 17 μM 2-Naph-DNB, respectively. Note the formation of evident bundles around the nucleus (arrowheads) in cells treated with 60 μM 2-Naph-DNB and the progressive lower tubular reorganization (see arrowheads) in cells treated with the lower concentrations. G, H, A549 cells treated with 1-Naph-NMCB administered at concentrations equitoxic to those used for 2-Naph-DNB (17.3 and 5.6 μM, respectively, see the corresponding panels D and F). Note that bundles are by far less prominent in G respect to D. I, resistant A549-T12 cells treated with 34 μM 2-Naph-DNB. As in the same cells treated with taxol, the rearrangement of microtubule in short organized structures disappeared.

taxol. This effect was in general concomitant with a decrease of S phase peaks (Table 2).

4. Discussion

As reported in a previous paper (Novi et al., 2004) the mechanisms of resistance of the cell lines used in our experiments are quite well characterized. All these cells were selected in vitro by prolonged exposure to the different selective anticancer drugs.

Briefly, the main mechanism of resistance of L1210/DDP cells consists of a decreased accumulation of cisplatin and a reduced DNA platination (Richon et al., 1987). The resistance of HCT-8/FU7dR cell lines was linked to the shortage of 5,10-methylenetetrahydrofolate, in turn due to a defect of its polyglutamylation (Aschele et al., 1992). In A2780/DX3 cells the drug resistance rests on an overproduction of Pgp 170, encoded by the MDR1 gene, and representing an ATP-pump devoted to the rapid efflux from the cells of the selective drug doxorubicin and of other unrelated anticancer drugs (Alaoui Jamali et al., 1989; Novi et al., 2004). Finally, in A549-T12 cells (whose growth requires a low concentration of taxol in the medium), the mechanism of resistance seems to be linked to the selection of point mutations on tubulins able to alter microtubule stability and increase their dynamics (Kavallaris et al., 1997; Gonçalves et al., 2001).

Our data suggest that, while all resistant cell lines do not present any pharmacologically significant cross-resistance between their selective drugs and 1-Naph-NMCB, A549-T12 cells show a significant cross-resistance towards the selective drug taxol and the nitrobutadiene compound 2-Naph-DNB. This evidence obviously lets us think that taxol and 2-Naph-DNB could share, although not necessarily, a common mechanism of resistance in these cells. If this is true then microtubules should be the target or, at least, one of the possible targets of 2-Naph-DNB. This hypothesis was easily verified by the analysis of the cell microtubule network after treatment with taxol or with our compound. The observation of the formation of bundles, similar enough to those observed after treatment with the microtubule-stabilizing agent taxol (Wehland et al., 1983; Liebmann et al., 1993; Kowalski et al., 1997), confirms that the microtubular sites responsible of taxol resistance in A549-T12 cells are directly or indirectly involved in the binding of 2-Naph-DNB on microtubules. On the contrary, 1-Naph-NMCB, which was active against A549-T12 resistant cells, does not show any gross bundling activity, similarly to the anticancer drug cisplatin whose mechanism of action is linked to its binding to DNA (data not shown). On the other hand, our data about the partial accumulation of cells in the G2/M phase of cell cycle in both A549 and A549-T12 cells strengthens this hypothesis, although the analysis of cell cycle phases may not be considered per se a specific test for sustaining it. To this regard it must be pointed out that the similar accumulation in the G2/M phase of the cell cycle of both A549 and A549-T12 cells is the result of equitoxic concentrations (IC_{50}) of anticancer compounds and that these treatment conditions were purposely applied in order to overcome the mechanism of resistance and bring into evidence any possible substantial difference in the alterations of cell cycle phases.

Table 2
Percentage of A549 and A549-T12 cells in the different cell cycle phases after treatment for 24 h with equitoxic concentrations (IC_{50}) of 1-Naph-NMCB, 2-Naph-DNB and taxol

Cell line	Cell cycle phases	Control	1-Naph-NMCB	2-Naph-DNB	Taxol
A549	G0/G1	41.8±5.5[a]	40.4±3.8	37.2±3.9	4.3±1.2[b]
	S	39.4±2.9	33.4±1.5[b]	34.5±7.0	23.7±1.3[b]
	G2/M	18.9±4.3	26.2±5.3[b]	28.3±4.9[b]	71.9±1.7[b]
A549-T12	G0/G1	51.1±4.4	52.0±8.3	51.1±4.0	7.8±3.1[b]
	S	28.4±4.5	24.2±6.4	22.4±8.6[b]	19.9±9.1
	G2/M	20.5±0.2	23.9±1.8[b]	29.5±0.9[b]	72.3±11.4[b]

[a] Mean±SD of 3 experiments.
[b] $P<0.05$, as determined by the Mann–Whitney test for not parametric data.

The search of new molecules with anticancer activity able to overcome intrinsic or acquired drug resistance remains one of the approaches for the development of new, more effective chemotherapeutic treatments. The fact that 1-Naph-NMCB may overcome some of the most common mechanisms of drug resistance selected in vitro by continuous exposure to drugs, with particular regard to the MDR that is relevant in some forms of clinical resistance phenomena, further contributes to make this compound a promising choice for a future development (Petrillo et al., 2008).

One additional significant result of this work resides in the observation that, while 1-Naph-DNB showed in previous experiments the same activity against A549-T12 cells and their sensitive counterpart (Novi et al., 2004), a relatively small change in its structure makes the modified molecule (i.e. 2-Naph-DNB) able to bind a new target site, activating possible new mechanisms of action. This is an important observation highlighting the fact that this new molecule could itself become a lead compound for the design of new antimicrotubule agents, thus opening a possible new line to anticancer research. For this reason and in spite of the cross-resistance observed, 2-Naph-DNB may be still considered another possible choice for the development of new active nitrobutadiene-derived anticancer compounds.

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