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Design, synthesis, and in vitro evaluation of new naphthylnitrobutadienes with potential antiproliferative activity: Toward a structure/activity correlation

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Abstract—On the grounds of previous encouraging results on the antitumor activity of (1*E*,3*E*)-1,4-bis(1-naphthyl)-2,3-dinitro-1,3-butadiene (**1**), we have designed and synthesized two new molecules [(1*E*,3*E*)-1,4-bis(4-carboxy-1-naphthyl)-2,3-dinitro-1,3-butadiene (**2**) and methyl (2*Z*,4*E*)-2-methylsulfanyl-5-(1-naphthyl)-4-nitro-2,4-pentadienoate (**3**)] characterized by a common naphthylnitrobutadiene array but with different structural properties, with the aim of approaching to some structure–activity correlation. When **2** and **3** were analyzed in vitro for their inhibition of cell proliferation and pro-apoptotic properties, the carboxyderivative **2** did not furnish appreciable results. In contrast, **3** (which contains only one of the two naphthylnitroethenyl moieties of the original compound **1**) showed remarkable activities in the range of micromolar concentrations (in six over eight cell lines its IC_{50}s are in the 1–3 μM range), with a significant improvement compared to **1**. In particular, **3** proved able to bind to DNA, to upregulate p53, to block cells in the G2/M phase of their cycle, and to induce apoptosis. Thus, very interestingly, the performance of **3** with respect to **1** shows that a single 1-(1-naphthyl)-2-nitroethene moiety is able to ensure better (on four out of eight of the cell lines tested) or comparable levels of activity. This result suggests that the 'molecular-simplification strategy' could furnish a useful instrument for future design in our antitumor research.

1. Introduction

The individuation of new molecular structures characterized by promising antiproliferative activities, in particular against most aggressive tumors, remains an important goal of pharmacological research, surely one to which continuous efforts are devoted worldwide.[1–4]

This is because the incidence of cancer (which is the main cause of mortality after cardiovascular diseases) has significantly increased in the last few years. Moreover, a remarkable number of tumor histotypes, such as those of stomach, colorectum, brain, pancreas, and lung, are still characterized by a very low prognosis and an unacceptably high mortality, because of unresponsiveness to the anticancer drugs generally utilized in clinics.[5–7]

Furthermore, different reasons linked to the global environmental modifications, today heavily dependent on the explosive progress of the social-economic conditions particularly in developed countries, have increased the risk of exposure to carcinogens, suggesting that the

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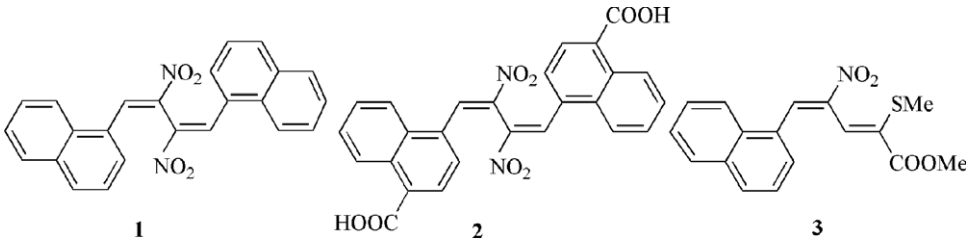


Figure 1. Molecular structure of compounds **1**–**3**.

incidence of cancer shall probably further speed-up in the next future.

Thus, one of the main tasks of today's research is that of increasing the available pool of original molecules characterized by: (i) an improved therapeutic index (which is generally low for traditional anticancer agents), obtained by means of a better balance between toxic and pharmacological effects, (ii) the ability to overcome the challenge of primary or acquired tumor resistance, and (iii) an enhanced spectrum of activity also against those tumor histotypes less responsive to classical as well as even to more recent anticancer drugs.

In previous papers[8–10] we have reported on the antiproliferative activity of (1*E*,3*E*)-1,4-bis(1-naphthyl)-2,3-dinitro-1,3-butadiene (**1**, Fig. 1). This molecule, derived from the initial ring-opening of 3,4-dinitrothiophene[11] and characterized by a new (symmetric) structure, has shown high activity in terms of inhibition of cell proliferation, cytotoxicity, induction of apoptosis, and selectivity for cell lines derived from otherwise unresponsive gastrointestinal tumors.

Such data suggest that the structure of **1** could represent a molecular arrangement capable to induce links to DNA, thus activating a sequence of effects eventually leading to the death of tumor cells or to the inhibition of their proliferation.[8–10]

In order to verify the correctness of our hypothesis and to better evaluate the effects of selected variations of the molecular structure on the pharmacological activity of **1**, we have synthesized the two new compounds **2** and **3** (Fig. 1). Herein we analyze their antiproliferative activity and their ability to form bifunctional links to DNA, to upregulate p53 protein, and to cause modifications of the cell cycle, with the particular aim to individuate possible novel mechanisms of action and to lay the basis for a first approach to a structure–activity correlation.

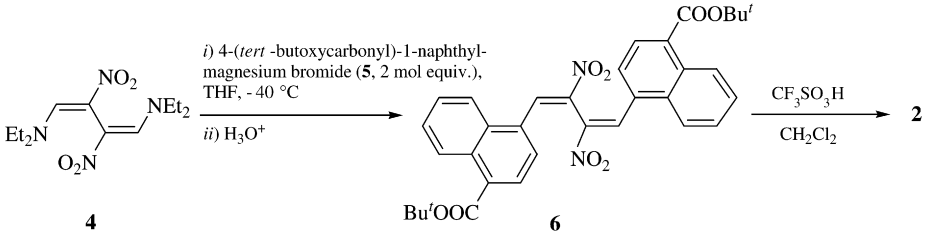
2. Results

2.1. Chemistry

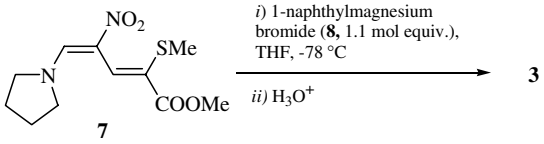
Likewise **1**, compounds **2** and **3** are stable, fully-characterized naphthylnitrobutadienes deriving from the products of initial ring-opening of nitrothiophenes (see Schemes 1 and 2 and Section 5).[11–14]

The symmetric derivative **2** differs from the 'lead' (**1**) because of the introduction of two carboxylic groups, that is, of hydrophilic functionalities which could (i) modify the activity by themselves (e.g., by means of different interactions with DNA) and/or (ii) allow a different administration methodology (directly after ionization or eventually by some modification of the carboxylic moiety). The synthesis of **2** has been achieved (Scheme 1) via the intermediate formation of **6** from **4**[11] by arylation with 4-*tert*-butoxycarbonyl-1-naphthylmagnesium bromide (**5**) followed by hydrolysis of the ester group with trifluoro methanesulfonic acid. It should be stressed that the arylation proceeds with complete retention of configuration at both double bonds of **4**, which is essential for the planned comparison between **2** and **1**.

On the other hand, compound **3** has been devised from **1** as an attempt to evaluate the effect of simplifying the 'double' 1-(1-naphthyl)-2-nitroethene moiety of the originally tested molecule to just 'one'. Interestingly it contains an ester group, that is, a potentially hydrophilic functionality, able to increase the water solubility. The synthesis of **3** is quite straightforwardly obtained (Scheme 2) by chemo- and stereo-selective arylation with 1-naphthylmagnesium bromide (**8**) of the product (**7**) of the ring-opening of 2-methoxycarbonyl-4-nitrothioph-



Scheme 1.



Scheme 2.

ene with pyrrolidine.[14] Once again, the stereoselectivity of the arylation step guarantees a more meaningful comparison between **3** and the original butadiene (**1**).

2.2. Biological results

2.2.1. Antiproliferative activity and induction of apoptosis. The mean concentration–response curves obtained from the treatment of cell lines with compound **3** showed that it was characterized by a significantly high antiproliferative activity. This was confirmed in terms of the micromolar range of IC_{50} values (range: 0.9 ± 0.2–12.1 ± 2.4 μM, median 2.2 μM; Table 1 and Fig. 2). In particular, P388, PA-1, A2780, HGC-27, and MDA-MB-231 showed the highest sensitivity to **3**.

A significantly different situation was found in the case of compound **2**, which showed instead a very low inhibition of cell proliferation, with IC_{50}s ranging from 129.3 ± 23.1 to 359.8 ± 19.5 μM, when tested against P388, Jurkat, PA-1, and A2780 cells (Table 1). These values were from 54- to 171-fold higher than those observed for **3** on the same cells. Because of such a low activity **2** was not further investigated.

As far as it concerns the cytotoxic activity of **3**, as evaluated by the Trypan blue dye exclusion assay, we applied two different concentrations (IC_{50} and IC_{75}), obtaining analogous results. In this context P388, A2780, Jurkat, and A549 proved to be the most sensitive cells to the cytotoxic activity of **3** (Table 1).

Finally, the 'simplified' derivative **3** showed a good activity, in some cases better than that observed for **1**,[8]

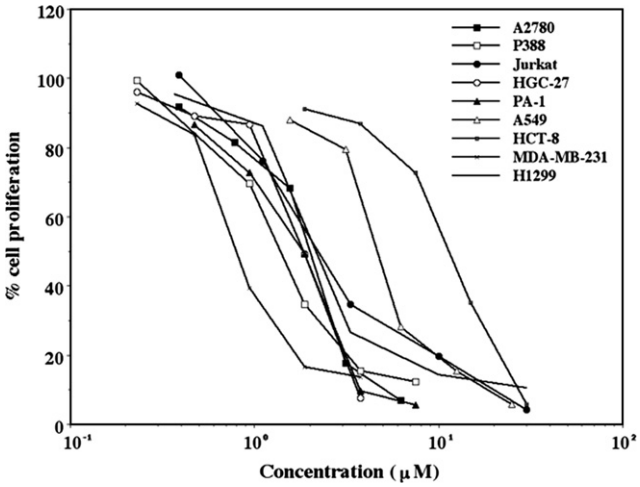


Figure 2. Antiproliferative activity of **3** on the different cell lines, as evaluated by the MTT assay. Each point represents the mean of 5–10 independent experiments.

also in terms of induction of apoptosis, as evaluated by the morphological analysis of nuclear segmentation. Also in this case results were particularly promising in the instance of P388, A2780, Jurkat, and A549 cells (Table 1).

2.2.2. Interstrand cross-links. On the grounds of its molecular structure, for the original compound **1** we postulated an interaction with the double strand of DNA. Actually, our recently published data[8–10] have confirmed this hypothesis, showing the formation of a noticeable amount of bifunctional adducts between **1** and DNA, which, in some cases, were more numerous than those caused by equimolar concentrations of cisplatin.

Since **3** shares with **1** a similar (although simplified) molecular structure it was tested for its ability to form interstrand cross-links (ISCL) using pure DNA isolated from P388 cells. Interestingly, **3** proved actually capable to form ISCL, although the percentage of ISCL found

Table 1. In vitro determination of inhibition of cell proliferation (IC_{50} values), cytotoxicity [Trypan blue (TB), % stained cells], and apoptosis [4′,6-diamidino-2-phenylindole (DAPI), % cells with segmented nuclei] after treatment with compounds **2** and **3**

Cell line	**1**	**2**	**3**		
	MTT[a,b] (IC_{50})	MTT[c] (IC_{50})	MTT[c] (IC_{50})	TB[d] (%)	DAPI[e] (%)
P388	2.1 ± 0.2	144.6 ± 7.9	1.4 ± 0.2	75 ± 4	27 ± 9
A2780	2.0 ± 0.2	359.8 ± 19.5	2.1 ± 0.7	55 ± 7	25 ± 9
PA-1	3.6 ± 0.3	129.3 ± 23.1	1.6 ± 0.3	22 ± 6	11 ± 5
Jurkat	2.8 ± 0.6	172.5 ± 14.0	3.2 ± 0.9	67 ± 14	14 ± 6
HGC-27	9.0 ± 1.0	ND	2.1 ± 0.2	21 ± 6	7 ± 3
MDA-MB-231	9.3 ± 1.0	ND	0.9 ± 0.2	16 ± 3	4 ± 2
HCT-8	9.8 ± 1.7	ND	12.7 ± 2.4	10 ± 3	5 ± 1
A549	14.3 ± 2.3	ND	6.0 ± 1.7	46 ± 12	60 ± 7
H1299	ND	ND	2.8 ± 0.7	14 ± 4	2 ± 1

[a] Mean ± SD of 3–8 IC_{50} values. All IC_{50} values are given in μM.
[b] See Ref. 8.
[c] Mean ± SD of 5–10 IC_{50} values.
[d] Mean ± SD of 5–11 values.
[e] Mean ± SD of 3–11 values.

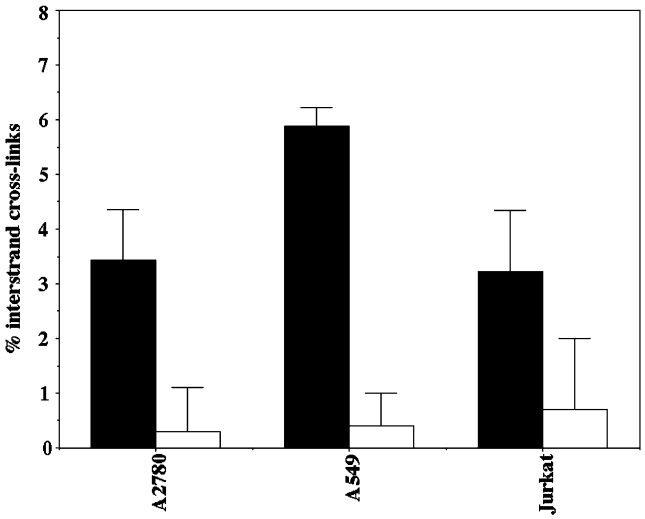


Figure 3. Histograms represent the % ISCL formed after exposure of A2780, A549, and Jurkat cells for 6 h at the IC_{50} of **3**, as calculated by the MTT assay. Data are expressed as means ± SD of 3–9 data.

was quite low (at 0.1 μM, 1.7 ± 0.4%; at 1 μM, 3.5 ± 0.10%). Similarly, results lower than those showed with compound **1** were obtained using DNA extracted from A2780, A549, and Jurkat cells treated with **3** (Fig. 3). Also in this case **3** confirmed not to form great amounts of ISCL.

2.2.3. Western blot analysis. Analysis by Western blot of p53 oncoprotein was performed on A2780, Jurkat, A549, MDA-MB-231, and PA-1 cells. Interestingly enough, in all of the cell lines **3** was able to upregulate p53 with a relative maximal increase of protein concentration ranging from +2% to +104%. As for **1**[8] and in spite of the limited number of time points used for this determination, our data reveal different kinetics for the development of maximal protein increase (Fig. 4a).

Finally, by means of control tests on A549 cells we could observe a correlation between two applied concentrations of **3** and the up-regulation of p53 (Fig. 4b).

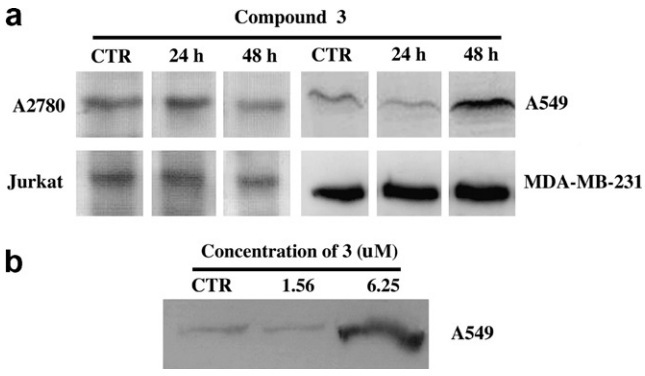


Figure 4. (a) Western blot analysis of p53 oncoprotein after exposure of cell lines for 24 and 48 h at concentrations of **3** corresponding at their IC_{50}s, as calculated by the MTT assay. Prestained molecular markers were always included as references. Control was harvested after 48 h. (b) Representative Western blot analysis of p53 after exposure of A549 cells at 1.56 and 6.25 μM of compound **3**.

Table 2. Percentage of A549 and Jurkat cells in the different cell-cycle phases after treatment with different concentrations of **3**

Cell line	Cell-cycle phase	Concentration of **3** (μM)			
		0	1.56	6.25	25
A549	G0/G1	49.8	46.5	49.0	41.2
	S	37.2	36.9	33.0	22.5
	G2/M	13.0	16.6	18.0	36.5
		0	0.37	3.33	15
Jurkat	G0/G1	40.2	31.1	41.3	51.2
	S	40.0	50.3	38.3	22.4
	G2/M	19.7	18.6	20.4	26.4

2.2.4. Cell cycle. The analysis of the cell-cycle phases on A549 and Jurkat cells showed that **3** was able to cause a partial block of cells in the G2/M phase of the cell cycle, with a concomitant decrease of cells in the S phase. This effect was strictly dependent on its concentration (Table 2).

3. Discussion

The synthesis of molecular structures characterized by specific activities against vital constituents of cells represents a good strategy for the development of new compounds with antiproliferative effects.

As outlined in the introduction, recent data[8–10] on the in vitro activity of the dinaphthyldinitrobutadiene **1** seem to support our hypothesis, based on the observation of the spatial arrangement of the molecule, of some effective binding to DNA as the primary cause of its antiproliferative activity. Accordingly, the structural modifications which have led to the synthesis of compounds **2** and **3** herein have been devised with the main aim of obtaining results useful to gain an approach to some structure–activity relationships: an outcome which could help in projecting further hits and hence in providing a new class of anticancer drugs to be confidently subjected to in vivo experiments.

As a matter of fact, the biological results reported above for compound **3** can be considered surely rewarding, as the molecule shows, with respect to **1**, a better antiproliferative activity, being at the same time significantly more effective also in terms of cytotoxicity and/or induction of apoptosis. Actually, **3** was able to form ISCL when tested on pure DNA, although its binding to DNA seems to be significantly lower than that observed for compound **1**[8] when tested on cell lines (which was in turn better than that observed for cisplatin in some cell lines).

Finally, **3** proves able to activate p53 in a concentration-dependent manner and with a different timing of up-regulation: this fact, together with the observation of a noticeable induction of nuclear segmentation, that is, the typical nuclear morphology of apoptotic cells, suggests that the molecule may effectively trigger apoptosis.

Actually, the real role of apoptosis in clinical setting has not been definitively clarified yet;[15–17] anyway, the fa-

vored hypothesis is that these physiological pathways leading to cell death may be important for the response to anticancer drugs in human patients.[18–20] Thus, anticancer compounds able to trigger the apoptotic machinery can be considered as good candidates for the development of new effective anticancer drugs.

Although not exceptional, the binding to DNA and the activation of p53 suggest that **3** might, at least in part, have DNA as target of its activity and that it could activate the apoptotic cascade starting from the damage (or imbalance) caused to this macromolecule. Moreover, the data concerning the modifications of the cell cycle induced by **3** demonstrate that the latter is able to block the cycle in the G2/M phase and that the extent of this damage in general correlates with the concentration applied and the percentage of apoptotic cells, as determined by the staining with DAPI for segmented nuclei and with Annexin-V (not shown). These data, together with the observation of p53 activation, still suggest a correlation between the damage caused by **3** to DNA or to other still unidentified molecular targets, the block at the G2/M cell-cycle checkpoint and the eventual apoptosis.

Recent unpublished data obtained using methylated lambda phage DNA treated with our nitrobutadiene compounds and cut by restriction enzymes of different specificity seem to confirm that compound **3** binds DNA less than compound **1** and, in part, at different cutting sites (data not shown). The quantitative differences in the ISCL formation but, above all, the slightly different modalities of DNA binding could let think of differences in the possible mechanism of action between compounds **1** and **3**. This is also suggested by the different block of cell-cycle phases observed after treatment with our compounds (G0/G1 vs G2/M phases). Obviously, these differences could also involve the presence of other still unidentified targets. To this regard experiments performed to identify a possible interaction of compound **3** with the cell microtubules failed to demonstrate an interaction with this cell constituent, as it was demonstrated for the isomer (1*E*,3*E*)-1,4-bis(2-naphthyl)-2,3-dinitro-1,3-butadiene of compound **1** (manuscript in preparation).

As far as it concerns the second structural modification planned, that is, the introduction of hydrophilic functionalities on the symmetric arrangement of **1**, the presence of the carboxylic groups in **2** seems only able to increase a little its water solubility, but clearly limited its antiproliferative activity, which was significantly lower than those observed for **3** as well as for the parent compound **1**.[8]

4. Conclusions

The good in vitro activity of the 'simplified' structure **3** described herein interestingly suggests that 'a single' 1-(1-naphthyl)-2-nitroethene moiety should be 'per se' able to ensure a good antitumor activity. The graphical abstract well illustrates the significance of the results obtained: it monitors the higher antiproliferative activity of **3** with respect to **1** versus cell lines PA-1, HGC-27, MDA-MB-231, and A549, and the comparable (or little better) activity versus cell lines P388, A2780, and Jurkat. Only versus the HCT-8 cell line **3** results a little less efficient than **1**. Moreover the growth curves evidence that in six cell lines out of eight **3** does work at low concentration (IC_{50}s lying within the 1–3 μM range). Interestingly enough, in the perspective of an approach to a structure–activity relationship the results herein, relevant to a 'structural simplification' from **1** (or **2**) to **3**, can be considered as a significant advancement in the understanding of the structural requisites needed for the antitumor activity as well as of the related mechanism of action, as far as this class of cytotoxic agents is concerned.

Moreover, the results obtained provide a further support to the efficacy of the molecular-simplification strategy[21,22] as a means for individuating pharmacologically significant structural arrays. The high activity observed in the instance of **3** indicates that a 'single' 1-(1-naphthyl)-2-nitroethenyl moiety is fundamentally responsible for the behavior of such a novel family of promising antitumor agents: it actually ensures good levels of activity in terms of inhibition of cell proliferation and/or cytotoxicity and/or induction of apoptosis.

Of course, such encouraging results (i) suggest the opportunity to subject **3** to in vivo tests and (ii) open the way to the study of the pharmacological activity of new, structurally-modified compounds (whose synthesis is in progress) in order to hopefully reach the definition of a structure–activity correlation.

5. Experimental

5.1. Chemistry

^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Mercury 300 spectrometer at 300 and 60 MHz, respectively; chemical shifts (TMS as internal reference) are reported as δ values (ppm). EI-MS spectra were recorded on a Thermo Finnigan Mat95XP apparatus; ESI-MS spectra were obtained on a micromass ZMD Waters instrument (30 V, 3.2 kV). Melting points were determined with a Büchi 535 apparatus and are uncorrected. All solvents were distilled before use. Petroleum ether and light petroleum refer to the fractions with bp 40–60 and 80–110 °C, respectively. Tetrahydrofuran (THF) was purified by standard methods and distilled over potassium benzophenone ketyl before use. Silica gel (63–200 mesh) was used for column chromatography. 1-Naphthylmagnesium bromide (**8**, ca. 0.25 M in THF) was prepared using standard procedures and titrated just before use. 4-Iodonaphthalene-1-carboxylic acid (**9**),[23] (1*E*,3*E*)-1,4 bis(diethylamino)-2,3-dinitro-1,3-butadiene (**4**),[11] and methyl (2*Z*,4*E*)-2-methylsulfanyl-4-nitro-5-pyrrolidino-2,4-pentadienoate (**7**)[14] were synthesized according to known procedures. All other commercially available reagents were used as received.

5.1.1. *tert*-Butyl 4-iodonaphthalene-1-carboxylate (10). *N,N′*-Carbonyldiimidazole (380 mg, 2.35 mmol) was added to a solution of 4-iodonaphthalene-1-carboxylic acid (**9**) (700 mg, 2.35 mmol) in dimethylformamide (4 mL) and the solution was heated at 40 °C for 2 h. Then 7,11-diazabicyclo[5.4.0]undec-11-ene (DBU) (0.35 mL, 2.35 mmol) and 2-methylpropan-2-ol (0.44 mL, 4.70 mmol) were added and the solution was heated at 40 °C for 4 h. The solution was diluted with diethyl ether, washed with 10% HCl, water, and 10% K_2CO_3, dehydrated (Na_2SO_4), and concentrated. After filtration on silica, 550 mg (66%) of **10** were obtained. Yellow oil; ^{1}H NMR ($CDCl_3$) δ 1.66 (9H, s), 7.55–7.66 (2H, m), 7.70 (1H, d, *J* 7.6 Hz), 8.12 (1H, d, *J* 7.6 Hz), 8.11–8.21 (1H, m), 8.75–8.80 (1H, m). Anal. Calcd for $C_{15}H_{15}IO_2$: C, 50.87; H, 4.27; I, 35.83%. Found: C, 50.73; H, 4.26; I, 35.70%.

5.1.2. (1*E*,3*E*)-1,4-Bis(4-*tert*-butoxycarbonyl-1-naphthyl)-2,3-dinitro-1,3-butadiene (6). Following a literature procedure,[24] 4-*tert*-butoxycarbonyl-1-naphthylmagnesium bromide (**5**) was prepared in THF (20 mL) by an exchange reaction from **10** (800 mg, 2.3 mmol) with freshly prepared isopropylmagnesium bromide (2.5 mmol). The THF solution of **5** was slowly added by syringe under argon and magnetic stirring to a suspension of **4** (300 mg, 1.0 mmol) in THF (20 mL), cooled to −40 °C. After 2 h the reaction mixture was poured into a mixture of dichloromethane and ice/water containing 2.5 mmol of HCl. After separation of the two layers, the aqueous phase was extracted with dichloromethane; the collected organic extracts were washed with water and dried over Na_2SO_4. Concentration under vacuum of the extracts gave a crude that was purified by column chromatography over silica gel (petroleum ether/dichloromethane gradients as eluent). The yield of **6** was 12%. Yellow solid, mp 208.7–209.2 °C (petroleum ether/dichloromethane); ^{1}H NMR ($CDCl_3$) δ 1.69 (18H, s), 6.69 (2H, d, *J* 8.4 Hz), 7.03–7.10 (4H, m), 7.30 (2H, t, *J* 7.4 Hz), 7.70 (2H, d, *J* 7.4 Hz), 8.33 (2H, d, *J* 8.8 Hz), 8.59 (2H, s); ^{13}C NMR ($CDCl_3$) δ 28.6, 82.3, 122.8, 124.9, 126.3, 127.1, 127.8, 128.1, 130.2, 130.4, 131.9, 132.4, 140.4, 144.1, 166.1. Anal. ($C_{34}H_{32}N_2O_8$) C, H, N. EI-MS: *m*/*z* (%) 596 (M^+, 15), 566 (10), 549 (12), 494 (11), 476 (14), 467 (28), 438 (44), 437 (32), 436 (41), 421 (30), 420 (84), 392 (32), 391 (20), 376 (21), 348 (30), 347 (53), 346 (72), 303 (37), 302 (100), 301 (65), 300 (68). ESI-MS: *m*/*z* 619.1 ($M+Na^+$), 596.2 (M), 595.2 (M−1). HRMS: Calcd for $C_{34}H_{32}N_2O_8$: 596.21587. Found: 596.21405.

5.1.3. (1*E*,3*E*)-1,4-Bis(4-carboxy-1-naphthyl)-2,3-dinitro-1,3-butadiene (2). Trifluoromethanesulfonic acid (0.4 mmol) was added to a solution of **6** (120 mg, 0.2 mmol) in dry dichloromethane (2 mL) under argon and magnetic stirring at room temperature. The resulting solution was kept in freezer overnight. The product (90%) was recovered by filtration. Light yellow solid, mp over 260 °C (petroleum ether/dichloromethane); ^{1}H NMR ($CDCl_3$) δ 3.48 (2H, s br), 6.93 (2H, d, *J* 8.2 Hz), 7.08 (2H, t, *J* 8.8 Hz), 7.23–7.35 (4H, m), 7.81 (2H, d, *J* 7.8 Hz), 8.35 (2H, d, *J* 8.8 Hz), 8.90 (2H, s); ^{13}C NMR (DMSO-d_6) δ 123.3, 125.3 (two signals not sufficiently resolved), 126,1, 127.2, 128.7, 129.4, 129.6, 130.0, 132.0, 141.4, 143.4, 167.6. Anal. ($C_{26}H_{16}N_2O_8$) C, H, N. EI-MS: *m*/*z* (%) 484 (M^+, 12), 454 (11), 438(16), 436 (18), 421 (18), 420 (49), 392 (19), 348 (25), 347 (47), 346 (80), 345 (16), 303 (38), 302 (100), 301 (62), 300 (74), 298 (17), 289 (23), 199 (44), 196 (20), 151 (41+21), 150 (47). ESI-MS: *m*/*z* 506.9 ($M+Na^+$), 482.8 (M−1). HRMS: Calcd for $C_{26}H_{16}N_2O_8$: 484.09067. Found: 484.09071.

5.1.4. Methyl (2*Z*,4*E*)-2-methylsulfanyl-5-(1-naphthyl)-4-nitro-2,4-pentadienoate (3). A solution of **7** (1 mmol) in THF (12 mL) was cooled to −78 °C under magnetic stirring. A small excess of a ca. 0.25 M solution of 1-naphthylmagnesium bromide (**8**, 1.1 mmol) in THF was slowly added by syringe and the reaction mixture left under stirring at the same temperature until disappearance of the substrate (ca. 45 min), monitoring the progress of the reaction by TLC. Quenching of the reaction was performed by pouring the final solution, with vigorous shaking, into a mixture of dichloromethane and ice/water containing 1.1 mmol of HCl. After extraction with dichloromethane, the organic phase was washed with water, dried with Na_2SO_4, and concentrated under reduced pressure. The product was recovered as a pure sample by chromatography of the residue on a silica gel column with a gradient of dichloromethane and petroleum ether as eluent (which also allowed to recover some unreacted **7**). The yield was 78% (93% on the reacted substrate). Yellow solid, mp 105.7–106.6 °C (light petroleum); ^{1}H NMR ($CDCl_3$) δ 2.01 (3H, s), 3.84 (3H, s), 7.43–7.48 (2H, m), 7.56–7.66 (3H, m), 7.89–7.97 (2H, m), 8.01–8.04 (1H, m), 8.78 (1H, s); ^{13}C NMR ($CDCl_3$) δ 16.26, 53.03, 123.78, 125.24, 126.79, 127.53, 128.27, 128.54, 128.97, 129.82, 131.55, 131.79, 133.54, 135.44, 137.35, 145.82, 164.33. Anal. ($C_{17}H_{15}NO_4S$) C, H, N. EI-MS: *m*/*z* (%) 329 (M^+, 1), 312 (4), 283 (29), 268 (18), 237 (18), 226 (17), 224 (29), 223 (49), 222 (17), 210 (18), 209 (26), 208 (100), 195 (24), 194 (19), 192 (16), 176 (24), 166 (17), 165 (75), 164 (37), 163 (24), 152 (19), 104 (34). ESI-MS: *m*/*z* 351.9 ($M+Na^+$). HRMS: Calcd for $C_{17}H_{15}NO_4S$: 329.07218. Found: 329.07209.

5.2. Biological assays

5.2.1. Dilution of the naphthyl derivatives for biological assays. Compound **3** was dissolved in DMSO at 60 and 100 mM concentration. These solutions were then further diluted with fetal calf serum to obtain the experimental concentrations used herein. The DMSO final concentration was always maintained lower than 0.2% (v/v). Compound **2** was dissolved in DMSO at 100 mM concentration. Following dilutions were obtained in phosphate-buffered saline.

5.2.2. Growth inhibition assay. Human [A2780 (ovary, carcinoma), Jurkat (T cell leukemia), PA-1 (ovary, teratocarcinoma), HGC-27 (stomach, carcinoma), MDA-MB-231 (breast, adenocarcinoma), HCT-8 (colon, adenocarcinoma), A549 (lung, carcinoma), H1299 (lung, carcinoma, kindly provided by Dr. K. Okaichi)] and murine [P388 (leukemia)] cells were plated at differ-

ent densities/well (range: 1500–5000/well) into 96-well microtiter plates (flat-bottomed for adherent cells and U-bottomed for non-adherent cells) for about 8 h. Compounds **2** and **3** were administered at the appropriate concentrations for a minimum of 5 concentrations (2- to 3-fold serial dilutions). Cells were treated in duplicate, while a final volume of 200 μL was reached in each well. After 72-h culture 50 μL of 3-(2,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT, Sigma, St. Louis, MO, USA) solution [2 mg/mL in phosphate-buffered saline (PBS)] was added to the wells and incubated at 37 °C for 4 h. Microplates were then centrifuged at 275g for 5 min and the culture medium carefully aspirated and replaced with 100 μL of 100% dimethylsulfoxide. Complete and homogeneous solubilization of formazan crystals was achieved after 20 min of incubation and shaking of well contents. The absorbance was measured on a 400 ATC microculture plate reader (SLT Labinstruments, Austria) at 540 nm.[25] IC_{50}s were calculated by the analysis of single dose response curves, each final value being the mean of 5–10 independent experiments.

5.2.3. Trypan blue (TB) dye exclusion assay and visualization of apoptotic cells by 4′,6-diamidino-2-phenylindole (DAPI) test. Cells were plated at different densities/well (range: 10,000–30,000/well) into 24-well microtiter plates (flat-bottomed) for about 8 h, then compound **2** was administered at its specific IC_{50} and IC_{75}, as determined with the MTT assay. After 72 h all cells were harvested and washed twice with cold PBS. After that, 50% of cells were concentrated to 100 μL normal saline and stained with 5 μL of TB (0.4% in 0.81% sodium chloride and 0.06% potassium phosphate dibasic). The percentage of stained dead cells was evaluated under the microscope. The remaining 50% of cells was fixed with 100 μL of 70% ethanol in PBS and maintained at 4 °C. Just before examination of fluorescence under the microscope, 5 μL of a solution of 10 μg/mL DAPI in water was added and the percentage of apoptotic segmented nuclei/cells stained with the fluorescent dye was evaluated.

5.2.4. Western blot analysis of p53. Human A2780, A549, Jurkat, and MDA-MD-231 cells were treated with their specific IC_{50}s for nitrobutadiene compound **3** (as calculated by the MTT assay). At 24- and 48-h culture aliquots of control and treated cells were harvested, washed twice with phosphate-buffered saline and treated with a lysis buffer [1% Triton X-100, 0.15 M NaCl, and 10 mM Tris (pH 7.4)] containing protease inhibitors (50 mg/mL phenylmethylsulfonyl fluoride and 2 mg/mL aprotinin) at 4 °C for 30 min. The protein concentration was determined by the Bradford method (Sigma Chemical Co., St. Louis, MO, USA). Equal amounts of total protein (30–60 μg) were separated on a 12% polyacrylamide gel (SDS–PAGE). After transfer onto a nitrocellulose membrane (Hybond C-Extra, Amersham Italia Srl, Milan, Italy), protein loading was checked by Ponceau S staining and non-specific binding was blocked overnight at 4 °C with 1% bovine albumin (Sigma) in Tris-buffered saline-Tween 20 [0.15 M NaCl, 10 mM Tris (pH 8.0), 0.05% Tween 20]. Blots were probed with anti-p53 DO-1 (1:2000, Santa Cruz, CA, USA) monoclonal antibodies. After incubation with horseradish peroxidase-conjugated antimouse IgG, bands were visualized by chemiluminescent detection (ECL™ Western blotting analysis system, Amersham Italia Srl) following the supplier's recommended procedures. Prestained molecular weight markers (New England Biolabs, Beverly, MA, USA) were used as reference. Alternatively, A549 (50 μg) cells were incubated for 72 h with different concentrations of **3**. Cells were then treated and p53 analyzed as described before.

5.2.5. Analysis of interstrand cross-link formation. Cells were treated for 6 h with the IC_{50} of compounds **1** and **3**, as calculated by the MTT assay. DNA was then extracted by the salting out technique[26] and dissolved in Tris–EDTA. Its yield and purity were determined by absorbance at 260 and 280 nm UV light. The % ISCL was also evaluated using pure DNA, extracted from P388 cells by the salting out technique (260/280 = 1.84) and dissolved in distilled water, as target. Two different concentrations (1.0 and 0.1 μM) of compound **3** were incubated with DNA (final concentration: 485 μg/mL) for 1 h at 37 °C. DNA was then precipitated with sodium acetate and ethanol and resuspended in Tris–EDTA. Ethidium bromide solution (10 μg/mL in 0.4 mM EDTA, 20 mM K_2HPO_4, pH 11.8, 3 mL) was added to 0.2 mL aliquots of DNA (20 μg) extracted from control and treated cells. The fluorescence was measured before and after heating at 90 °C for 10 min (Perkin-Elmer LS-5B spectrofluorimeter; excitation wavelength, 525 nm; emission wavelength, 580 nm). The % ISCL was determined according to the formula:

$$(ft - fn)/(1 - fn) \times 100$$

where ft and fn were the fluorescence after denaturation divided by the fluorescence before denaturation of treated (ft) and control (fn) samples.

5.2.6. Analysis of the cell cycle. A549 and Jurkat cells were analyzed for the effect of **3** on cell-cycle phases. To this end cells ($1–2 \times 10^6$) were treated for 48 h with three different concentrations of **3**, harvested, washed twice with cold PBS, and fixed in 70% ethyl alcohol at −20 °C overnight. Following fixation, cells were centrifuged, washed with PBS, and incubated at room temperature for 20 min with propidium iodide (PI) staining solution (50 μg/mL PI, 0.05% Triton X-100, 20 mg/mL RNase A in PBS). Cells were subjected to flow cytometric analysis of DNA content using FACSort flow cytometer (BD Biosciences, Mountain, View, CA). The percentages of cell cycle distribution were calculated by ModFit LT computer program.

5.2.7. Statistical analysis. Unpaired Student's *t* test was used for statistical analysis of data.

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