Strategies for improving the water solubility of new antitumour nitronaphthylbutadiene derivatives†

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Different nitronaphthylbutadienes have been previously proved to have antitumour activity. The main drawback of these derivatives is their low water solubility. With the aim of facilitating the administration of these new drugs we have synthesized the hexyl (2*Z*,4*E*)-2-methylsulfanyl-5-(1-naphthyl)-4-nitro-2,4-pentadienoate analogue (**1-Naph-NHCB**) which is demonstrated to be easily included into cyclodextrins and/or entrapped into liposomes. Its antitumour activity was revealed to be almost comparable with that of the previously studied methyl analogue ester (**1-Naph-NMCB**). On the other hand, *in vitro* studies with different cancer cell lines showed that the cytotoxic activity of both **1-Naph-NMCB** and **1-Naph-NHCB** were fully preserved and in some cases also enhanced when entrapped into liposomal carriers.

Introduction

Drug administration and delivery represent fundamental steps for the transition from a bioactive product to a commercial drug.[1] This is especially true for antitumour agents, whose administration usually requires direct or indirect solubility in physiological solutions to allow an intravenous injection often carried out by continuous infusion.

To achieve this goal several approaches have been attempted. Covalent modifications have allowed increasing the hydrophilic character of lipophilic drugs by insertion of appropriate groups on the drug skeleton.[2] The use of sequestering agents, such as cyclodextrins (CDs)[3] or liposomes,[4] has recently received much attention due to the double advantage of increasing the water solubility and ensuring a time-controlled release of the drug.

In the last several years our research groups have been involved in a program aimed at finding new antitumour compounds characterized by significant and/or particular specificity, able to induce lower drug resistance phenomena, and with a good therapeutic index in order to improve the response rates and increase the patients' survival. We have tested[5–8] different compounds characterized by the naphthylnitro-butadiene or ethene group which has been recognized[6–8] (by exploiting the "molecular-simplification strategy") to be a molecular arrangement effective for binding to DNA and therefore probably responsible for the observed antitumour activity. The first preclinical results on compound (1*E*,3*E*)-1,4-bis(1-naphthyl)-2,3-dinitro-1,3-butadiene (**1-Naph-DNB**, see Fig. 1) showed a significant activity towards several malignant cell lines and a good *in vivo* activity as well as low toxic effects.[5,6] Nevertheless the development of **1-Naph-DNB** as a drug has been limited due to its very low solubility in water and in physiological solution. A step towards an increase in water solubility has been previously made by synthesizing methyl (2*Z*,4*E*)-2-methylsulfanyl-5-(1-naphthyl)-4-nitro-2,4-pentadienoate (**1-Naph-NMCB**, see Fig. 1),[7] an analogue that keeps only one of the two naphthyl moieties of **1-Naph-DNB**, but whose calculated log *P* value [ACD/Labs version 6.00] is just 3.2 ± 0.4, well below that of the parent compound (*i.e.* calculated log *P* for **1-Naph-DNB** is 6.8 ± 0.3). Interestingly enough, the calculated log *P* value of **1-Naph-NMCB** stays in the range proposed by Lipinski in his rule-of-five.[9] Moreover also the other parameters of the rule (**HB**-donor and **HB**-acceptor centres, molecular weight) are satisfied by **1-Naph-NMCB**. As a matter of fact, **1-Naph-NMCB**, even if not completely soluble in water, showed an *in vitro* antiproliferative activity (on four out of the eight cell lines tested) more significant than that found for the original **1-Naph-DNB**.[6–8]

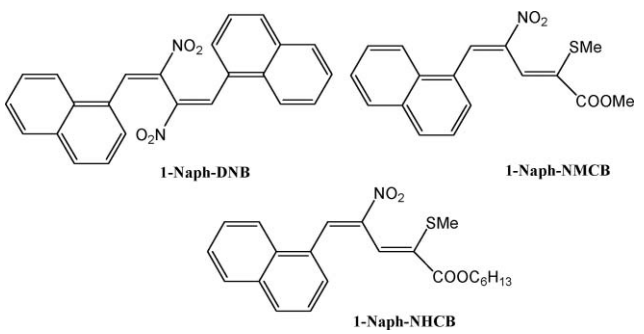

Fig. 1 Molecular structure of **1-Naph-DNB**, **1-Naph-NMCB**, and **1-Naph-NHCB**.

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† Electronic supplementary information (ESI) available: Positive ion ESI MS mass spectra of the complexes of **1-Naph-DNB** with β-CD and of **1-Naph-DNB** with DMβCD; titration of **1-Naph-DNB** with β-CD or with DMβCD. See DOI: 10.1039/c0ob00493f

In the present study we intend to improve further the administration of the investigated molecules by increasing the water solubility of these series of derivatives by following a different strategy. Instead of following the covalent functionalization approach we have exploited the ability of some macromolecules or supramolecules to incorporate or complex **1-Naph-DNB** or **1-Naph-NMCB**. With this aim our attention has been addressed to two different guest systems: the "including" cyclodextrins (CDs) and the "incorporating" liposomes. Both of them have been already introduced as efficient drug carriers in medical practice. In addition, we also tested the hexyl (2*Z*,4*E*)-2-methylsulfanyl-5-(1-naphthyl)-4-nitro-2,4-pentadienoate analogue (**1-Naph-NHCB**), whose calculated log *P* value [ACD/Labs version 6.00] is 5.8 ± 0.4. The reason for synthesizing **1-Naph-NHCB** is two-fold: (i) the presence of an hexyl alkyl chain should favour the inclusion into CDs as it has been widely demonstrated[10] that β-cyclodextrins can include hexyl alkyl chains efficiently and selectively and (ii) the hydrophobicity of the guest molecule can control the efficiency of its integration into the bilayer, a hydrophobic molecule being directly integrated into the bilayer upon dispersion with lipids.[11]

CDs can accommodate water insoluble drugs within their hydrophobic cavities to form water soluble inclusion complexes. These inclusion complexes increase solubility and dissolution rate, decrease volatility, increase stability and generally pharmacological activity.[12] The dissociation of drug/CD inclusion complexes *in vivo* takes place either because of dilution or because other blood components displace the included drug. The released drug is then metabolized as free drug while the cyclodextrin moiety is excreted through the kidneys.[13]

Liposomes represent as well useful means for chemical and medical applications. They are vesicles produced from natural non-toxic phospholipids in the presence or in the absence of cholesterol.[14] Because of their size, biocompatibility and capacity to incorporate both hydrophilic (in the aqueous core) and lipophilic (in the bilayer) compounds, liposomes are promising systems for drug delivery and the potential of liposome-mediated delivery of various drugs to cells has long been recognized.[15] Liposome properties vary substantially with lipid composition, size, surface charge, protocol of preparation, and depend strongly on the cell investigated. Pagano *et al.*[16] demonstrated that vesicles are incorporated into cells both by endocytosis and by non-endocytotic mechanisms. The former mechanism predominates in the case of liposomes prepared from neutral phosphatidylcholine, while non-endocytotic fusogenic pathways predominate in the uptake of negatively charged vesicles composed of phospholipids that are "fluid" (phosphatidylserine/phosphatidycholine) at 37 °C.[17] The mechanism of fusion between cytoplasma membrane and liposomes has been particularly investigated in order to avoid the lysosomal hydrolysis and therefore the decomposition of hydrolysis-sensitive substances such as proteins or nucleotides.[18] The coating of liposomes with poly(ethylene glycol) chains inhibits[19] protein adsorption and opsonization, thereby avoiding or retarding liposome recognition by the RES, improving their stability and lengthening their half-lives in circulation. These so called stealth liposomes have been successfully introduced in clinical trials in antitumour therapy. Anyway non-PEGylated liposomes have also been approved for clinical use (for example in metastatic breast cancer).[20]

Results

CD complex formation

Several experiments were performed both in the gas phase and in solution in order to verify the possible formation of inclusion complexes of **1-Naph-DNB** and **1-Naph-NHCB** with cyclodextrins (β-CD and DMβCD).

Electrospray ionization mass spectrometry (ESI-MS) is a suitable technique to study the complexation of relatively apolar compounds with cyclodextrins. In fact the ions formed in solution can be transferred[21,22] into the gas phase without breaking the non-covalent interactions which are the predominant forces in "host–guest" supramolecular systems. ESI-MS analysis in the gas phase revealed the presence in the studied aqueous solutions of host/guest complexes between the cyclodextrins and both the investigated substrates.

In Fig. 2 the spectrum of a solution containing 68.5 μM of **1-Naph-NHCB** and 2.5 mM of β-CD is reported. The spectrum is characterized by a peak corresponding to the 1 : 1 host–guest complex (*m*/*z*: 1536.4) and a more intense peak assignable to the 1 : 2 complex (*m*/*z*: 2671.9).

Interestingly, ESI experiments performed on **1-Naph-DNB** in the presence of β-CD or DMβCD just after mixing did not evidence any 1 : 2 complex (see Fig. S1†). On the other hand, spectra recorded once the samples were left mixing for 20 min (*i.e.* the same experimental conditions adopted for UV-vis determinations, see below) revealed the presence of 1 : 2 complexes as well (see Fig. S2†). Therefore, due to the capability of ESI-MS to resolve[23] the dynamic processes occurring in complex supramolecular systems, it is essential to allow the system to reach the equilibrium before performing ESI-MS experiments.

The formation of inclusion complexes between **1-Naph-DNB** or **1-Naph-NHCB** and cyclodextrins in aqueous solution has also been studied by UV-visible spectroscopy. The prerequisite for the use of the spectroscopic techniques for complex investigation is the change in the spectral characteristics of the guest molecule once included in the host. The UV-vis spectroscopy is therefore a method widely used to study the formation of inclusion complexes for its high sensitivity and versatility.[24,25]

For both substrates a bathochromic shift could be observed for the lower energy band upon addition of cyclodextrin. A red shift from 387 to 393 nm was monitored for **1-Naph-DNB** upon increasing β-CD concentration from 0.2 to 10 mM, while λ changed from 379 to 385 nm upon increasing DMβCD concentration from 0.2 to 8 mM. In the case of **1-Naph-NHCB** a red shift from 368 to 387 nm was observed upon passing from 0.1 to 7 mM β-CD. The lack of any isosbestic point in the absorption spectra on increasing the concentration of cyclodextrin is indicative of the fact that there are at least two complexes of different stoichiometry in solution thus confirming the ESI-MS evidence.

The stoichiometric ratios and binding constants were deduced by fitting the changes of absorbance in the presence of increasing amounts of β-CD and DMβCD to the following sequential complexation of cyclodextrin molecules:

$$S + CD \leftrightarrows S^*CD: K_{11} = [S^*CD]/([S]\,[CD]) \quad (1)$$

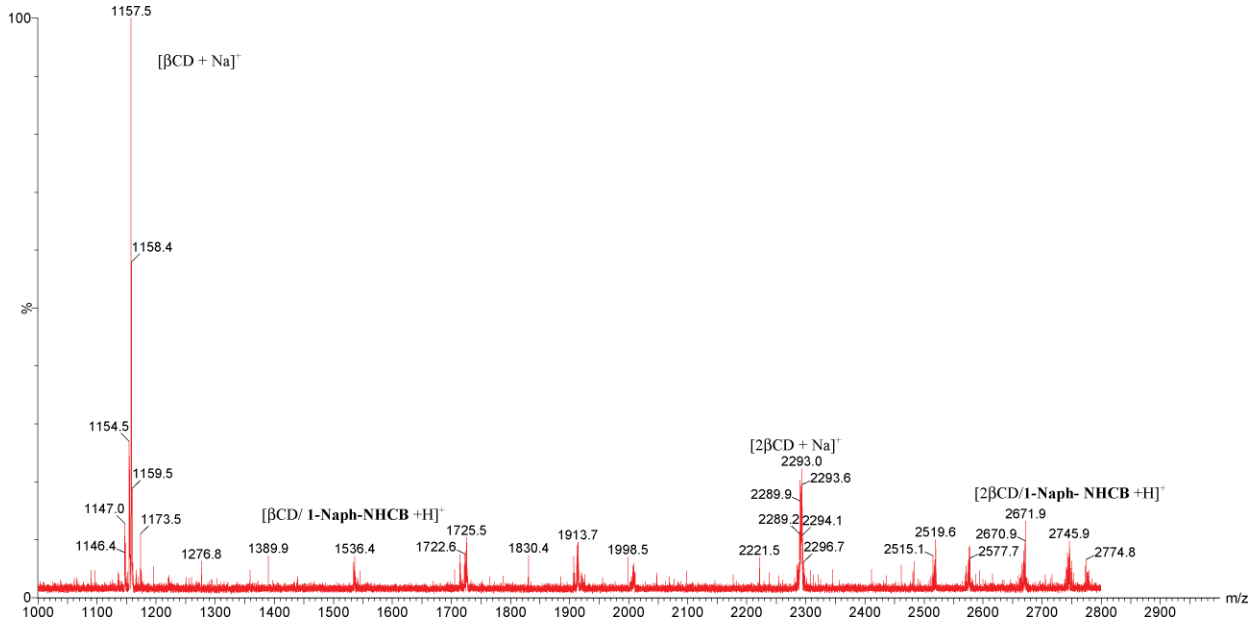


Fig. 2 Positive ion ESI MS mass spectra of the complexes of **1-Naph-NHCB** with β-CD.

$$S^*CD + CD \leftrightarrows S^*(CD)_2: K_{12} = [S^*(CD)_2]/([S^*CD]\,[CD]) \quad (2)$$

with S being the investigated nitronaphthylbutadiene, K_{11} = binding constant of the 1 : 1 complex and K_{12} = binding constant of the 1 : 2 complex. The experimental data have been fitted into the following eqn (3),[25,26]

$$\frac{\Delta A}{b} = \frac{S_t \beta \Delta\varepsilon [CD]^2}{1 + \beta [CD]^2} \quad (3)$$

where $\Delta A = A - A_0$, A being the absorbance at each CD concentration and A_0 the absorbance in the absence of CD; b = length of the cell in cm; S_t = initial concentration of substrate; $\beta = K_{11}K_{12} = [S^*(CD)_2]/([S]\,[CD]^2)$; $\Delta\varepsilon = \varepsilon - \varepsilon_0$, ε being the molar absorptivity of the associated and ε_0 that of the free substrate, respectively.

Fig. 3 reports the titration of **1-Naph-NHCB** with increasing amounts of β-CD. The corresponding titration curves for **1-Naph-DNB** on addition of β-CD and DMβCD are reported in the ESI (Fig. S3 and S4†).

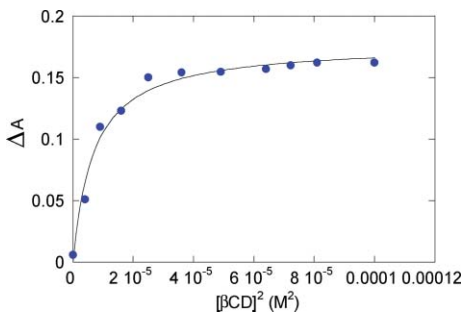


Fig. 3 Titration of **1-Naph-NHCB** with β-CD at λ_{max} 387 nm. The experimental data are fitted into eqn (3).

From a best fit of experimental A values to eqn (3) the β values reported in Table 1 were obtained. The values of β show that the affinity of the investigated substrates for the cyclodextrin cavity is high for both substrates and both the investigated cyclodextrins. The structural features of **1-Naph-DNB** and **1-Naph-NHCB** are compatible with the inclusion of both aromatic rings or both the aromatic ring and the alkyl chain within the hydrophobic cavity, respectively. The β values measured for **1-Naph-DNB** are almost ten times higher than that for **1-Naph-NHCB** thus highlighting the higher affinity of cyclodextrin for the naphthyl with respect to the alkyl moiety in agreement with the previously published[27] 10 times higher affinity of β-CD for anthracene with respect to 1-hexanol.

Table 1 Binding constants of **1-Naph-DNB** and **1-Naph-NHCB** with β-CD and DMβCD

Host/guest	β/M^{-2}
β-CD/**1-Naph-DNB**	$1.58(\pm0.32) \times 10^6$
DMβCD/**1-Naph-DNB**	$9.46(\pm1.45) \times 10^5$
β-CD/**1-Naph-NHCB**	$1.47(\pm0.21) \times 10^5$

Liposome encapsulation

The encapsulation efficiency (e.e.) of the investigated derivatives was calculated as follows (eqn (4)):

$$\text{e.e.} = 100 \times \frac{\text{mass of incorporated derivative}}{\text{mass used for liposomes preparation}} \quad (4)$$

The liposomal entrapping efficiency calculated by HPLC analysis and the mean size of liposomes obtained by scattering measurements are reported in Table 2.

The spontaneous leakage of carboxyfluorescein (CF) from the liposomes was monitored spectrofluorimetrically.[28,29] The initial fluorescence intensity of CF was very low, due to self-quenching of the dye. Once liposomes start to release the dye by leakage, the emission intensity of the fluorescent probe increases (because of the dilution-dependent de-quenching) according to a first order kinetics (eqn (5)):

Table 2 Encapsulation efficiency of **1-Naph-NMCB** and **1-Naph-NHCB** in POPC liposomes

Derivative	$[POPC]_{initial}$/μM	$[derivative]_{initial}$/μM	Formulation[a]	Mean size/nm	e.e.[b] at t_0	e.e.[b] at t_{180} (e.e. at t_{360})	Measured [derivative]/μM at t_0
1-Naph-NHCB[c]	300	100	**1** - SUV	184 ± 1	13 ± 1	14 ± 1 (19± 1)	0.25
1-Naph-NHCB[c]	600	100	**2** - SUV	150 ± 1	21 ± 2	21 ± 1 (24± 1)	0.90
1-Naph-NHCB[d]	600	100	**2** - SUV	150 ± 1	57 ± 6		57
1-Naph-NMCB[d]	600	100	**2** - MLV	ND[e]	40 ± 2		40

[a] SUV: small unilamellar vesicles; MLV: large multilamellar vesicles. [b] t_0 corresponds to HPLC data obtained from the liposome formulations after extrusion and filtration on Sephadex G-75, t_{180} and t_{360} correspond to the HPLC data obtained from the liposome formulations filtered after 180 and 360 min from their extrusion, respectively. [c] Samples used for the kinetic measurements after dilution. [d] Samples used for the cell proliferation tests. [e] ND: not determined.

Table 3 Triton X-100-induced release of CF from POPC/**1-Naph-NHCB** liposomes

[Triton X-100]/μM	POPC[a] 10^{-5} k (s^{-1})	Formulation **1** 10^{-5} k (s^{-1})	Formulation **2** 10^{-5} k (s^{-1})
0	6.61(±0.05)	3.38(±0.30)	3.00(±0.10)
24	21.1(±0.1)	4.75(±0.03)	3.74(±0.10)
32	25.2(±0.1)	6.17(±0.04)	4.36(±0.10)
44	43.7(±0.2)	20.9(±0.1)	16.5(±0.1)
52	66.8(±0.5)	30.6(±0.2)	25.4(±0.1)
60	83.3(±0.1)	59.5(±0.5)	42.6(±0.2)
64	104(±1)	88.8(±0.4)	61.4(±0.5)

[a] Data from ref. 29.

$$d[CF]/dt = k_{obs}[CF] \quad (5)$$

The rates of release of CF were also determined at different concentrations of the non-ionic single chain surfactant, Triton X-100, employed as a destabilizing agent.

The pseudo-first order rate constants, k_{obs}, can be related to the concentration of Triton X-100 following eqn (6):

$$k_{obs} = k[\text{Triton X-100}]^n \quad (6)$$

where n represents the order of the CF release process.

The kinetic data for the spontaneous leakage of CF from the liposomes and the release of dye induced by the addition of Triton X-100 are shown in Table 3.

Biological results

Since the solubility in aqueous solution and in fetal calf serum of the inclusion complex of β-CD and of DMβCD containing **1-Naph-DNB** or **1-Naph-NHCB** was very low, not exceeding 1 μM, none of these preparations could be used for cell proliferation assay.

1-Naph-NHCB was shown to be characterized by a significantly high antiproliferative activity in a micromolar range of IC_{50} values (range: 1.98 ± 0.54 to 3.85 ± 0.19 μM). In particular P388, A2780 and MDA-MB-231 showed the highest sensitivity to **1-Naph-NHCB** (See Table 4). As compared to the previously studied **1-Naph-DNB** and **1-Naph-NMCB** derivatives, **1-Naph-NHCB** showed a really good activity, in some cases (HGC-27, MDA-MB-231 and A549) significantly higher than that of **1-Naph-DNB**. The inclusion into liposomes does affect only moderately the activity of **1-Naph-NHCB** increasing its IC_{50} in A2780, PA-1 and A549 cells and lowering it in HCT-8 cells. On the other hand, the inclusion into liposomes increases substantially the activity of **1-Naph-NMCB** particularly on five (P388, A2780, HGC-27, HCT-8 and A549 cells) out of the eight cell lines tested (Table 4).

Discussion

The investigated **1-Naph-DNB** and **1-Naph-NHCB** showed a good affinity for both β-CD and DMβCD with relatively high binding constants. From a combined study using both ESI-MS and UV/vis measurements the prevailing inclusion complexes appear to be those with a derivative : CD stoichiometry of 1 : 2. Unfortunately, the solubility of these inclusion complexes in aqueous solutions and in fetal calf serum is limited (1 μM) and none of these samples could be used for cell proliferation measurements. Nevertheless the high binding constants obtained for the studied nitronaphthylbutadienes with cyclodextrins could pave the way for investigating their affinity with more hydrophilic cyclodextrins.

Interestingly, **1-Naph-NMCB** and **1-Naph-NHCB** can instead be easily entrapped into liposomes so as to obtain aqueous solutions of these derivatives compatible with cell proliferation measurements. HPLC data point out that a certain amount of **1-Naph-NHCB** is lost during liposomal preparation (*i.e.* gel permeation) but liposomes obtained by following formulation **2** can entrap, at the maximum investigated concentration, about 60% of the initially added amount of **1-Naph-NHCB**. The presence of **1-Naph-NHCB** in the bilayer strongly increases the stability of POPC vesicles, even in the presence of a destabilizing agent such as Triton X-100 (see Table 3, liposomes prepared with formulation **2**), thus potentially favouring a long circulation of the drug in the body fluids and consequently accumulation at target sites. This stabilizing activity of **1-Naph-NHCB** on the liposomes is particularly intriguing. The drug itself could be efficiently exploited for increasing the circulation lifetime of the liposome cargo avoiding the drawbacks of stealth liposomes, which beneficially accumulate at tumour growth sites but are often unable to realize acceptable drug release profiles.[30] This stabilizing activity depends on the entrapped amount of **1-Naph-NHCB**. Table 3 shows that the stabilizing activity of liposomes obtained by formulation **1**, characterized by an amount of **1-Naph-NHCB** three times lower than that of liposomes prepared by following formulation **2**, is strongly reduced with respect to the former investigated system. Unfortunately, it was not possible

Table 4 *In vitro* determination of inhibition of cell proliferation (IC_{50} values (μM)) after treatment with compounds **1-Naph-DNB**, **1-Naph-NMCB**, and **1-Naph-NHCB**

Cell lines	**1-Naph-NHCB**	n^a	Liposomes/**1-Naph-NHCB**	n^a	*p* values	**1-Naph-NMCB**	n^a	Liposomes/**1-Naph-NMCB**	n^a	*p* values	**1-Naph-DNB**
P388	1.98 ± 0.54	12	2.21 ± 0.46	4	NS	1.44 ± 0.22	7	0.69 ± 0.18	5	<0.001	2.08 ± 0.17
A2780	2.03 ± 0.20	4	2.33 ± 0.13	4	<0.05	2.11 ± 0.74	12	1.05 ± 0.15	4	<0.02	1.95 ± 0.21
PA-1	3.85 ± 0.19	4	4.86 ± 0.77	6	<0.05	1.60 ± 0.33	8	2.38 ± 0.65	7	<0.02	3.64 ± 0.27
Jurkat	3.54 ± 0.84	8	4.10 ± 1.30	6	NS	3.16 ± 0.85	9	2.46 ± 0.78	8	NS	2.83 ± 0.57
HGC-27	3.71 ± 0.67	6	3.40 ± 0.26	6	NS	2.10 ± 0.16	10	1.14 ± 0.08	4	<0.001	8.96 ± 1.02
MDA-MB-231	2.27 ± 0.29	4	2.03 ± 0.22	4	NS	0.89 ± 0.16	8	1.86 ± 0.09	6	<0.001	9.33 ± 0.99
HCT-8	39.02 ± 3.66	6	19.49 ± 4.50	7	<0.001	12.66 ± 2.43	12	9.68 ± 2.12	6	<0.05	9.84 ± 1.68
A549	6.16 ± 1.13	6	8.07 ± 0.31	4	<0.02	5.95 ± 1.67	10	3.46 ± 0.70	5	<0.01	14.25 ± 2.25

[a] *n* is the number of performed experiments.

to incorporate **1-Naph-NMCB** into unilamellar liposomes as it tended to accumulate onto the polycarbonate filters of the extruder during the preparation. Nevertheless it was possible to entrap **1-Naph-NMCB** into multilamellar liposomes with the molar ratio of formulation **2**. A final and reproducible concentration of 40% of the initially added **1-Naph-NMCB** was recovered into the sample. It is recognized that multilamellar vesicles are usually less effective or more toxic[31] than small unilamellar vesicles in pharmaceutical liposomal formulations. In the present case multilamellar vesicles appear to be more effective than unilamellar liposomes in cell proliferation measurements, but very likely this enhanced activity is due to the relatively higher anticancer activity of **1-Naph-NMCB** when compared to that of **1-Naph-NHCB** (see Table 4).

As far as cell proliferation measurements are concerned, liposomes performed quite well as drug carriers of **1-Naph-NMCB**. In particular, a mean increase of inhibition of cell proliferation of about 43% (as evaluated on IC_{50}s) was observed in the case of P388, A2780, HCT-8, A549 and HGC-27 cell lines (out of the eight cell lines tested) compared to the non-incorporated derivative. On the other hand no significant improvement of cell proliferation was detected when unilamellar liposomes embedding **1-Naph-NHCB** were investigated (see Table 4). Such encouraging results suggest the opportunity to perform *in vivo* tests on liposomes embedding the investigated derivatives. As a matter of fact, *in vivo* activity depends on several variables: the concentration of loaded drug, the duration of drug exposure and therefore the clearance of the lipid nanocarrier, the rate of release of the drug from the carrier system, and the clearance of the free (unencapsulated) agent once released from the liposome. Therefore, the loaded amount being equal, not all liposomes are equivalent, both in terms of the stability of the liposome and the ability of the drug to diffuse through the liposomal membrane. A factor that influences the pharmacokinetics of liposomal nanocarrier systems is the size, even if conflicting data have been published with either smaller liposomes taken up much more slowly from the blood circulation[32] or with an *in vitro* uptake negatively correlated with the liposomal size.[33] For this reason it would be wise to study in the near future the *in vivo* activity of both small unilamellar and multilamellar liposomes, as it is generally proved that the former SUV liposomes are most stable, retaining in the presence of serum a high percentage of the entrapped drug, with respect to the latter MLV liposomes.[31]

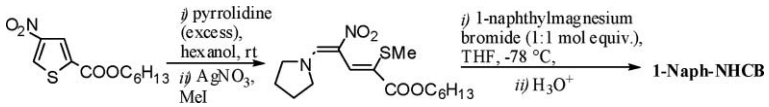
Conclusions

This study highlights both the good antitumour activity of the investigated derivatives and the possibility of significantly increasing their solubility in aqueous or physiological solutions. This is a crucial point when we consider that the effective cell antiproliferative concentration of **1-Naph-NHCB** almost coincides with its water solubility. Indeed, the administration of the free drug appears not to be compatible with retention of antitumour activity due to the extreme dilution conditions (*i.e.*, "sink" conditions) encountered by the drug following physiological administration. In particular **1-Naph-NHCB** is quite active and its activity is retained when embedded into unilamellar liposomes. Interestingly enough, the encapsulation of **1-Naph-NMCB** into multilamellar liposomes seems able to strongly enhance its antitumour activity.

Experimental section

Chemistry

1-Naph-DNB[34] and **1-Naph-NMCB**[7] have been synthesized according to known procedures. **1-Naph-NHCB** has been synthesized according to Scheme 1 and its properties are reported below. β-CD and DMβCD, CF, Triton X-100 are commercial samples from Fluka and Sigma-Aldrich, respectively. 1-Palmitoyl-2-oleoyl-*sn-glycero*-3-phospho-choline (POPC) was purchased from Avanti Polar Lipids (Alabaster, AL). A Jasco FP-6200 spectrofluorimeter and a Varian Cary 1E spectrophotometer were employed for the determination of kinetic rate constants and binding constants at 25.0 ± 0.1 °C. The osmolarity of the liposomal samples was checked using a microsmometer Advanced Instruments Mod.



Scheme 1 Synthesis of **1-Naph-NHCB**.

3300. ^{1}H NMR and ^{13}C NMR spectra of **1-Naph-NHCB** were recorded on a Varian Mercury 400 spectrometer. ESI-MS spectra were obtained on a ZMD Micromass single quadrupole mass spectrometer.

Hexyl (2*Z*,4*E*)-2-methylsulfanyl-5-(1-naphthyl)-4-nitro-2,4-pentadienoate (1-Naph-NHCB). The hexyl (2*Z*,4*E*)-2-methylsulfanyl-5-(1-naphthyl)-4-nitro-2,4-pentadienoate was synthesized and purified according to the procedure adopted for the synthesis of **1-Naph-NMCB**.[7] Yellow solid, mp 123.2–123.9 °C; λ_{max}(MeOH)/nm 221 (log ε 5.53); δ_H (400 MHz; $CDCl_3$; Me_4Si) 0.85–0.92 (3H, m), 1.26–1.40 (6H, m), 1.65–1.74 (2H, m), 2.01 (3H, s), 4.23 (2H, t, *J* 6.8), 7.42–7.48 (2H, m), 7.55–7.65 (3H, m), 7.88–7.96 (2H, m), 8.02 (1H, *J* 8.0), 8.76 (1H, s); δ_C (400 MHz; $CDCl_3$; Me_4Si) 13.94, 16.22, 22.47, 25.55, 28.44, 31.33, 66.39, 123.79, 125.20, 126.76, 127.49, 128.33, 128.58, 128.95, 129.42, 131.57, 133.55, 135.13, 137.79, 146.02, 163.93; ESI-MS: *m/z* 399.9 $(M+H)^+$, 421.9 $(M+Na)^+$, 821.4 $(M+Na+H)^{2+}$; HRMS: calcd. for $C_{22}H_{25}NO_4S$: 399.15043, found: 399.15051.

ESI-MS measurements

The experiments were carried out in water–acetonitrile mixtures (1 : 1, v/v) to which 5% of methanol was added. The *host/guest* molar ratio is about 100 for all experiments; the instrument was set with the following parameters: capillary voltage 3.3 kV, cone voltage 30 V and desolvation temperature 150 °C. The sample solutions were introduced at flow rate of 15 μL min^{-1}. The spectra were performed just after mixing the guest to the cyclodextrin host (t_0, see Fig. S2) and after 20 min of mixing (t_{20}, see Fig. S1).

UV/vis measurements

The stoichiometric ratios and the binding constants of the inclusion complexes were derived from the changes of absorbance in the presence of increasing amounts of β-CD and dimethyl-β-CD (DMβCD).

1-Naph-DNB (35 μM) has been studied in the presence of β-CD (0.2 to 10 mM) and DMβCD (0.2 to 8.0 mM), while **1-Naph-NHCB** (10 μM) in the presence of β-CD (0.1 to 7.0 mM). The solutions were left to equilibrate at 25.0 ± 0.1 °C for 20 min before recording the spectra.

The fitting of eqn (3) for the measurement of β has been performed by using UV-vis data recorded at λ 387 nm for the binding of **1-Naph-NHCB** to β-CD, and at λ 223 nm for the binding of **1-Naph-DNB** to β-CD or to DMβCD.

Preparation of liposomal formulations

Small unilamellar vesicles (SUV) composed by POPC/**1-Naph-NHCB** were prepared by adding the appropriate aliquot of a 13.1 mM POPC chloroform solution to 80 μL of a 2.5 mM chloroform solution of **1-Naph-NHCB**. Two different phospholipid/**1-Naph-NHCB** molar ratios were used for the formulation of liposomes. Formulation **1** was prepared by incorporating 25% mol of **1-Naph-NHCB**; formulation **2** was prepared by incorporating 14% mol of **1-Naph-NHCB**. The organic solvent was removed by evaporation under vacuum to obtain a dry film. The film was kept at 4 °C overnight and then 2 mL of a phosphate buffer made of 174 mM NaCl, 105 mM Na_2HPO_4, 20 mM KH_2PO_4 (pH 7.40 and 578 mOsm) were added to obtain a theoretical concentration of 0.3 mM POPC and 0.1 mM **1-Naph-NHCB** in the case of formulation **1** or 0.6 mM POPC and 0.1 mM **1-Naph-NHCB** in the case of formulation **2**.

The resulting multilamellar vesicle dispersion was extruded through polycarbonate filters (Osmonics, pore size 220 nm) mounted in an extruder from Lipex Biomembranes, Vancouver BC, Canada to obtain a homogeneous and monodisperse population of large unilamellar vesicles.

The same procedure was adopted for entrapping **1-Naph-NMCB**. However, the latter molecule proved to be less soluble in the liposomal bilayer and it was not possible to perform, in this case, the final extrusion process. The liposomes obtained by using the molar ratio of formulation **2** are in this case multilamellar (MLV) and non homogeneous liposomes whose dimensions vary in the range 0.5 to 10 μm[35] depending on the lamellarity of the liposome. For these latter liposomes only cell proliferation tests were performed due to the difficulty in handling and performing fluorescence measurements with such non-homogeneous samples.

In order to separate the unentrapped nitronaphthylbutadiene derivative from the loaded liposomes the vesicle solution was purified by gel-filtration on a Sephadex G-75 column.

Encapsulation efficiency

The amount of **1-Naph-NMCB** and **1-Naph-NHCB** incorporated within the liposomal bilayer was assessed by HPLC on purified vesicles after their disruption with isopropanol (vesicles dispersion/isopropanol 1/1). All analyses were carried out on a Spectra System P-200 pump equipped with a Spectra System UV6000LP photo-diode array detector and a computer integrating apparatus. The column was a Vydac C4, the mobile phase consisted of a filtered and degassed 60/40 acetonitrile–water mixture. The flow rate was kept constant at 0.5 mL min^{-1}.

In the case of **1-Naph-NHCB** the encapsulation efficiency measurements were repeated after 180 and 360 min (t_{180} and t_{360}, respectively) from liposome preparation in order to investigate the stability of both formulations after the extrusion.

In the case of HPLC measurements over the time, the sample was filtered on a Sephadex G-75 column before performing the following analysis in order to get rid of the amount of **1-Naph-NHCB** that could have been released in the bulk aqueous phase. All the measurements were repeated three times.

Dimensional analysis

The size of the extruded POPC/**1-Naph-NHCB** liposomes was evaluated before the addition of isopropanol by dynamic laser light scattering, using the Stokes–Einstein relationship for the calculation of the hydrodynamic radius from Brookhaven (90PLUS BI-MAS) digital correlator at a scattering angle of 90° equipped with a 10 mW He–Ne laser at wavelength of 660.0 nm. The size of the POPC/**1-Naph-NMCB** was not evaluated due to the lack of homogeneity of the corresponding multilamellar samples.

Kinetic measurements

The stability of the large unilamellar vesicles was determined by following the leakage of the 5(6)-carboxyfluorescein (CF) entrapped at its self-quenching concentration into the vesicles during

the hydration of the dry film at room temperature under stirring. A similar investigation was not attempted for the multilamellar, and therefore non-homogeneous, samples due to the fact that liposomes of different size are characterized by different properties and different rate of CF leakage.[28,36]

After the extrusion and the filtration on a Sephadex G-75 column the liposomal dispersion was run through a Sephadex G-50 column to remove the unentrapped dye from the loaded vesicles.

The spontaneous leakage of CF from the liposome was investigated at 25.0 ± 0.1 °C by using 490 and 516 nm as the excitation and emission wavelength, respectively, and a band pass of 5 nm. For the kinetic measurements the concentration of POPC was kept constant at 13 μM; the initial concentration of **1-Naph-NHCB** was 4.3 μM in the samples prepared by following formulation **1** and 2.0 μM in the samples prepared by following formulation **2**, respectively.

Dilution of the naphthyl derivatives for biological assays

Compounds **1-Naph-NMCB** and **1-Naph-NHCB** were dissolved in DMSO at 100 mM. These solutions were then further diluted with fetal calf serum to obtain the experimental concentrations (10×) used herein. The DMSO final concentration was kept lower than 0.2% (v/v). When encapsulated both preparations were diluted at the proper concentrations in phosphate buffered saline (PBS) at pH 7.4.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) 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), and murine P388 (leukemia) cells were plated at different concentration/well (range: 1000–5000 cells/well) into 96-well microtiter plates (flat-bottomed for adherent cells and U-bottomed for non-adherent cells) centrifuged and incubated for about 6–8 h. The choice of the cells was dictated either by the necessity to test cells sensitive to different anticancer drugs and because they were already used[5,6,7] for screening on structurally related compounds.

Compounds **1-Naph-NMCB** and **1-Naph-NHCB**, either embedded with liposomes (measured sample concentrations are reported in the last column of lines 3 and 4 of Table 2) or free, were then added at the appropriate concentrations for a minimum of 5 concentrations (2–3 fold serial dilutions).

Cells were treated in duplicate reaching a final volume of 200 μL in each well. After 3 days culture 50 μL MTT (Sigma, St. Louis, MO, USA) solution (2 mg mL^{-1} in PBS) was added to the wells and incubated at 37 °C for 4 h. Microplates were centrifuged at 275 g for 5 min and the culture medium aspirated and replaced with of 100% dimethyl sulfoxide (100 μL). Complete solubilization of formazan crystals was achieved after 30 min incubation at 37 °C and shaking of well contents. The absorbance was measured on a 400 ATC microculture plate reader (SLT Labinstruments, Austria) at 540 nm.[37]

IC_{50}s were calculated by the analysis of single dose response curves, each final value being the mean of 4–10 independent experiments.

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