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Diarylsemicarbazones: synthesis, antineoplastic activity and topoisomerase I inhibition assay

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Abstract

A series of diarylsemicarbazones was synthesized and tested against human neoplastic cell lines. The more active members have a 1-naphthyl ring at the carbamidic nitrogen, and chloro, dimethylamino or nitro group substituents at the benzylidene moiety. None of these showed affinity to DNA. One of the more active compounds was tested as a topoisomerase I inhibitor and showed a potent effect. SAR studies demonstrated linear correlation between lypophilicity and activity on the most sensitive lines and a definite conformational shape for antineoplastic action. © 1999 Elsevier Science S.A. All rights reserved.

Keywords: Diarylsemicarbazones; Synthesis; Antineoplastic activity; Topoisomerase I inhibition

1. Introduction

It is known that a partial modification of some carcinogenic molecular structures can decrease their carcinogenic effect and can raise the cytostatic effect, turning such compounds into potential therapeutic agents. The antileukemial amsacrine, which derives from 9-aminoacridine, provides a good example of this. Semicarbazide, as well as hydrazine itself, has proved mutagenic and carcinogenic activities [1]. Several authors have explored these effects [2]. Hitherto no systematic investigation of semicarbazide derivatives has been developed in this application area, as have Sartorelli and co-workers with thiosemicarbazides [3].

In a previous paper from this laboratory, the synthesis of an aryl-alkylaminosemicarbazone series (Fig. 1) and the activity against cell lines have been described [4]. These compounds were not active, except for the one with $n=3$ and a 4-nitrophenyl moiety, which showed cytostatic activity against CNS cell lines ($-\log GI_{50}=4.26$) and breast ($-\log GI_{50}=4.19$).

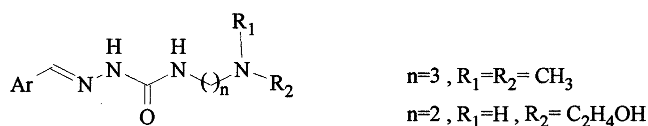

Fig. 1. Synthesis of an aryl-alkylaminosemicarbazone series.

Prompted by the above and assuming that the 4-nitrophenylsemicarbazone group could be a basic pharmacophore for this type of compound, a second series in which the aliphatic chain in the carbamidic nitrogen is replaced by aromatic moieties was planned (Fig. 2).

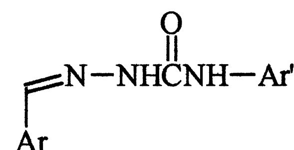

Fig. 2.

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Table 1
2-Arylmethylene-*N*-arylhydrazinecarboxamides: physical data and antineoplastic activity

Comp.	Ar	Ar′	Yield (%) [a]	M.p. (°C)	MG MID values [b]		
					Log GI_{50}	Log TGI	Log LC_{50}
1	Ph	Ph	32 [c]	164–166 [e]	−4.00	−4.00	−4.00
2	$4\text{-NO}_2C_6H_4$	Ph	20 [c]	225–227	−4.00	−4.00	−4.00
3	1-Naph	Ph	24 [c]	144–146	−4.00	−4.00	−4.00
4	6-MeO-2-Naph	Ph	20 [c]	215–217	−4.00	−4.00	−4.00
5	5-NO_2-1-Naph	Ph	20 [c]	200–205	−4.91	−4.48	−4.11
6	Ph	1-Naph	20 [c]	187–189 [f]	−4.42	−4.07	−4.01
7	$4\text{-NO}_2C_6H_4$	1-Naph	25 [c]	>250 [g]	−4.61	−4.21	−4.04
8	$4\text{-N(Me)}_2\text{-}C_6H_4$	1-Naph	68 [c]	186–189	−5.29	−4.36	−4.02
9	1-Naph	1-Naph	50 [c]	241–243	−4.73	−4.14	−4.02
10	6-MeO-2-Naph	1-Naph	70 [c]	202–204	−4.47	−4.11	−4.03
11	4-ClC_6H_4	1-Naph	59 [c]	216–218	−5.21	−4.37	−4.12
12	4-MeOC_6H_4	1-Naph	85 [c]	204–206	−4.69	−4.13	−4.01
13	4-ClC_6H_4	2-Naph	55 [d]	216–218	−4.28	−4.04	−4.00
14	$4\text{-NO}_2C_6H_4$	2-Naph	60 [d]	215–220	−4.42	−4.08	−4.01
15	4-ClC_6H_4	6-MeO-2-Naph	32 [d]	184–186	−4.11	−4.00	−4.00
16	$4\text{-NO}_2C_6H_4$	6-MeO-2-Naph	25 [d]	228–230	−4.41	−4.06	−4.01
17	1-Naph	$4\text{-NO}_2C_6H_4$	82 [d]	212–214	−4.80	−4.20	−4.01
18	2-Naph	$4\text{-NO}_2C_6H_4$	80 [d]	217–219	−4.67	−4.21	−4.02
19	6-MeO-2-Naph	$4\text{-NO}_2C_6H_4$	81 [d]	214–216	−4.79	−4.37	−4.09

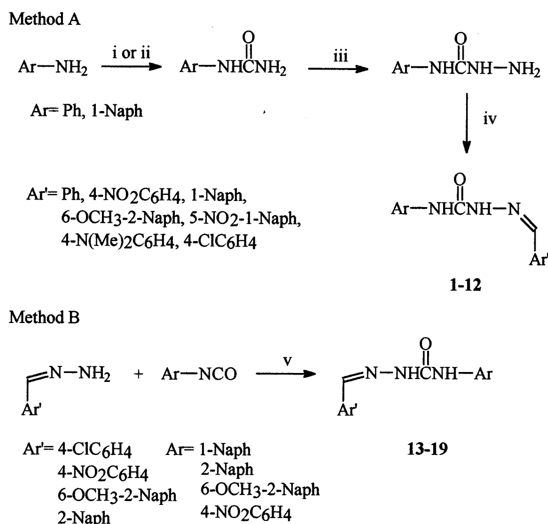
[a] Compound **1** was recrystallized from benzene and **3** from toluene. Compounds **2** and **4–12** were triturated with EtOH while **13–19** were washed with EtOH because they decomposed on heating.
[b] The letters MG MID refer to the term 'mean-graph midpoint'. It shows the average sensitivity of all cell lines towards the test agent a +50% (GI, grown inhibition), 0% (TGI, total grown inhibition) and −50% (LC, lethal concentration) of tumor growth.
[c] Method A.
[d] Method B.
[e] Lit. m.p. 180°C [14].
[f] Lit. m.p. 200–201°C [14].
[g] Lit. m.p. 257–258°C [14].

Nineteen compounds were synthesized (Table 1) and evaluated in vitro against human tumor cell lines by the National Cancer Institute (USA).

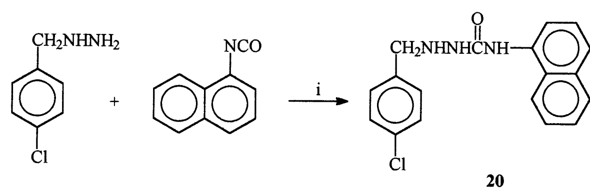
2. Results and discussion

2.1. Chemistry

The target compounds were obtained by two different synthetic procedures. The first started by treatment of an arylurea with hydrazine to afford the respective aryl hydrazinecarboxamides which were finally condensed with several substituted aromatic aldehydes, compounds **1**–**12** (Method A, Scheme 1). *N*-(1-Naphthyl)hydrazinecarboxamide could not be prepared by the same procedure because 2-naphthylamine reacted with urea to produce only *N*,*N*′-di-2-naphthylurea, which resulted in being very stable under hydrazinolysis conditions. Because of this, 2-naphthyl derivatives were obtained by means of the reaction of 2-naphthylisocyanates with the appropriate aromatic aldehyde monohydrazone, compounds **13**–**16** (Method B, Scheme 1). 2-Naphthyl and 6-methoxy-2-naphthylisocyanates were easily obtained via the respective acylazides by Curtius' procedure [5]. Compounds **17**–**19** were prepared by this latter method employing 4-nitrophenylisocyanate also as there is no reaction between *N*,*N*′-di-(4-nitrophenyl)urea and hydrazine.



Scheme 1. (i) KCNO/AcOH or (ii) H_2NCONH_2/py/reflux; (iii) $H_2NNH_2{\cdot}H_2O$/EtOH/reflux; (iv) Ar′CHO/EtOH/reflux; (v) Benzene/r.t.



Scheme 2. (i) Benzene/r.t.

Extensions of the SAR for this class of compound also required the preparation of two analogs of the most active compounds. One of them, **20**, where the semicarbazone double bond carbon–nitrogen was replaced by a saturated semicarbazide, was obtained by means of the condensation of 1-naphthylisocyanate with 4-chlorobenzylhydrazine (Scheme 2). The other, **21**, in which a methylene group was between the carbamidic nitrogen and the 1-naphthyl moiety, was synthesized from 1-naphthylmethylamine by Method A (Fig. 3).

Semicarbazones are capable of displaying *E*/*Z* isomerization pertaining to the carbimino double bond. When ^{1}H NMR spectra were recorded in DMSO dissolution and 3 h after incubation at 37°C, no spectral changes were noted. The stereochemistry of the carbimino group in different semicarbazones with anticonvulsant activity was shown by X-ray crystallography to have only the *E* configuration [6].

Analytical and spectroscopic IR and ^{1}H NMR data of this series agreed with the proposed structures and are described in Section 4.

2.2. Pharmacology and SAR

The diarylsemicarbazones and semicarbazide derivatives were evaluated by the NCI following the known screening program [7] for in vitro cytotoxicity and cytostaticity against 60 human tumor cell lines. The most simple structures (**1**–**4**) displayed little cytostatic activity ($-\log GI_{50} = 4.00$) while the remaining members of the series showed cytostatic activity values from 4.11 to 5.29 (Table 1). CNS, renal and colon cell lines were the most sensitive. These results show that the minimally active structure is benzyliden-1-naphthylsemicarbazide. Molecular modeling with AM1 semi-empirical calculations [8] estimates a minimum energy conformation where the plane of the phenyl moiety and the plane of the 1-naphthyl ring are arranged at an angle which changes little depending on the *para* phenyl substituting nature, whereas the carbimino group has the *E* configuration in all series compounds. Replacement of the carbimino moiety with methylene-hydrazino (a change from semicarbazone to semicarbazide), compound **20**, substantially modified the conformation and dramatically reduced activity ($-\log GI_{50} = 4.13$). When a methylene group separates the 1-naphthyl moiety, compound **21**, the conformation also changed and consequently the activity diminished ($-\log GI_{50} = 4.10$) (Fig. 4). The minimal structure shapes of the most active compounds **7**, **8** and **11** can be superimposed with good similarity. These compounds with close activity have a nitro, chloro and dimethylamino group, respectively, in the 4-position of the phenyl ring. It discounts any variations on account of basicity or dipolar moment caused by these groups, suggesting a possible relationship between activity and lypophilicity of such groups. Provided that renal cell lines resulted in being the most sensitive to the cytostatic effect of this series, the correlation between their activity and lypophilicity determined from the retention time on a reverse phase column was studied. Taking into account compounds **1**–**19**, the correlation coefficient is 0.56; if seven particular compounds with little or high activity are selected, this correlation coefficient rises to 0.85 (Fig. 5 and Eq. (1)).

$$\log(GI_{50}) = 0.419 \text{ retention time} + 3.096 \quad (1)$$

$n = 7 \; r = 0.85$

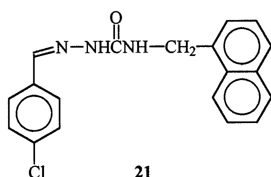


Fig. 3. Structure of 2-(4-chlorophenylmethylene)-*N*-(1-naphthylmethyl)hydrazinecarboxamide (**21**).

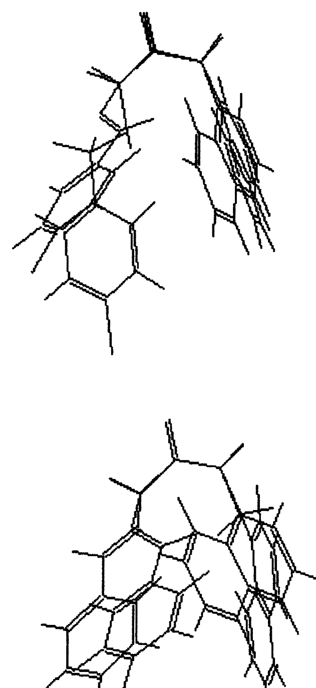


Fig. 4. Superimposed images of compounds **11** and **20** (top) and **11** and **21** (bottom).

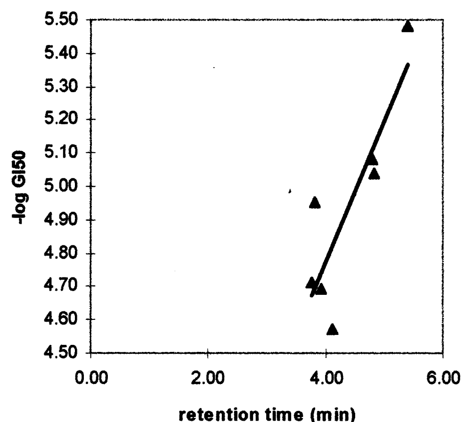


Fig. 5. Activity on renal cell lines vs. lypophilicity (**5**, **6**, **7**, **8**, **11**, **12** and **17**).

In order to establish a possible active mechanism for these semicarbazone derivatives, their binding to calf thymus DNA was determined by an UV method [9]. The degree of interaction was expressed by means of the ratio between the final absorbance area (a_{24}) and that of the compound at equal concentration (a_0). Values of 1 or greater indicate a total lack of affinity and value 0 that the entire compound is bound to DNA. This classical procedure was improved by means of slow rotating DNA–drug mixture stirring, in a 5:1 ratio, for 24 h and it was validated by repeating assays with recognized intercalating agents (*m*-AMSA and mitoxantrone) and a compound which binds closely in the minor groove (bis-benzimide [10], Hoechst no. 33258). Under such experimental conditions, these compounds rendered values of 0.54, 0.00 and 0.57 respectively. None of the series compounds showed affinity to DNA.

Redox properties of the most active compounds were examined by cyclic voltammetry. Except for the reductive nitro peak of compound **7**, the patterns were alike and indicated an irreversible redox process.

2.3. Topoisomerase I inhibition assay

Several major forms of human cancer express high levels of topoisomerase enzymes and respond with extreme sensitivity to topo I or II highly specific inhibitors and also to a few specific products that perform as mixed topoisomerase I/II inhibitors [11]. Because of this, a representative compound of the series (**11**) was assayed with topo I inhibitor.

While studying the in vitro effect of **11** on the relaxation of supercoiled pHOT1 catalyzed by human topoisomerase I, it was found that the relaxation activity was inhibited by exposure to the drug at doses of 100, 10 and 1 μg/ml (Fig. 6(a), Lanes 4–6). The topoisomerase I poison, camptothecin, was also examined

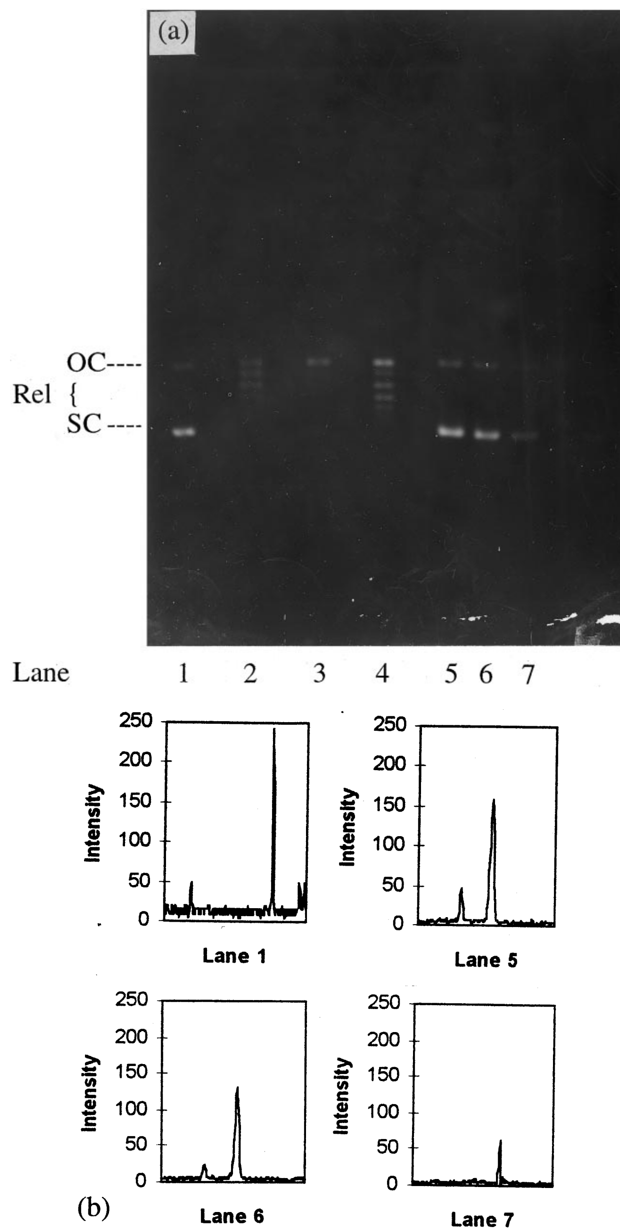


Fig. 6. Inhibition of relaxation of supercoiled DNA by compound **11**. Supercoiled pHTO1 DNA (Lane 1) was incubated with topoisomerase I in the absence (Lane 2) or presence of the drug: 100, 10 and 1 μg/ml in Lanes 5–7. Lane 3: camptothecin 0.1 mM (control). Lane 4: Relaxed plasmid DNA marker (control). The DNA was analyzed by agarose gel electrophoresis. The gels were stained with ethidium and photographed. OC, open circular; rel, DNA relaxed; SC, supercoiled DNA. (b) Measurement of supercoiled DNA band intensity in each lane of the gel. The areas under the peaks were integrated and % inhibition of relaxation of supercoiled DNA by compound **11** were obtained as described in Section 4.

and the results are shown in Fig. 6(a) (Lane 3). As expected, camptothecin evidenced an increase in OC DNA and an inhibition of conversion of supercoiled substrate to relaxed DNA.

Agents capable of stabilizing the topoisomerase cleavage complex have been termed 'topoisomerase poisons'. This distinguishes them from catalytic inhibitors,

which act by interfering in the binding of topoisomerases to DNA, in the formation of the active intermediate, or by trapping the topoisomerase on the DNA once the reaction is complete [12]. The results obtained suggest that the drug may belong to the latter class.

For quantitative analysis, the photographic negatives were scanned and the bands analyzed with an image analysis program. Inhibition of relaxation activity was determined as described in Section 4. 88, 42 and 33% inhibitions for 100, 10 and 1 μg/ml of drug (Fig. 6(b)) were found. Thus, inhibition of relaxation activity by the drug occurs in a dose-dependent manner. The minimal concentration necessary to inhibit 50% of the topoisomerase I activity (IC_{50}) was found to be 27 μg/ml (95% confidence interval: 14–69 μg/ml).

3. Conclusions

Introduction of the 1-naphthyl ring at the carbamidic nitrogen produces compounds that possess marked antineoplastic activity. The more active members have the phenyl ring substituted at the 4-position with groups that increase the lypophilicity. The conformation of all active compounds is quite similar. Structural changes that modified such shape deeply diminish activity.

Apparently, this series of compounds neither interact with DNA nor have redox properties so it suggests that oxy radical generation may be an active mechanism. A possible alternative could be some specific enzyme inhibition such as gyrases. Compound **11** showed a potent inhibition effect against topo I, but if it is non-specific this compound could also be a topo II inhibitor.

Compounds **7** (NSC 674493) and **11** (NSC 680933) were selected by the NCI for further in vivo investigation.

4. Experimental

4.1. Chemistry

Melting points were determined with a Büchi melting point apparatus in open capillaries and are uncorrected. IR spectra were recorded on KBr discs, using a Jasco A-200 spectrophotometer. ^{1}H NMR spectra were obtained in DMSO-d_6 on a Bruker 200 MHz spectrophotometer and chemical shifts are reported relative to TMS. HPLC was performed on a Konik KNK-500-A series liquid chromatograph. A Konik model UVIS 204 spectrophotometer was used as a detector. Peaks were recorded on a Konik Data Jet integrator. A reverse-phase C_{18} column was used. Samples for injection were dissolved in acetonitrile at a concentration of 1×10^{-4} M. The mobile phase was acetonitrile:water 70:30 and the flow rate was 1 ml/min. UV spectra were registered on a Jasco 7850. TLC was performed using silica gel sheets with a fluorescent indicator. Elemental analyses were in good agreement with our results.

4.2. N-Phenyl and N-(1-naphthyl)hydrazine-carboxamide

These compounds were prepared according to the literature [13,14] and were used in the next step without further purification.

4.3. Preparation of arylisocyanates: general procedure

2-Naphthyl and 6-methoxy-2-naphthylisocyanate were synthesized from the corresponding acid chloride by Curtius degradation, as follows.

Naphthoylchloride (27 mmol) dissolved in *i*-PrOH was added dropwise to a solution of NaN_3 (2.39 g, 37 mmol) in 10 ml water with stirring at 0–5°C. Then it was allowed to warm to room temperature (r.t.) and the solid product formed was extracted with benzene. The organic layer was dried ($MgSO_4$) and then it was heated for 2 h to thermally decompose the acylazide. The solvent was removed and 2-naphthylisocyanate was purified by vacuum distillation (77% yield), b.p. 125–127°C, 3 mmHg and 6-methoxy-2-naphthylisocyanate by vacuum sublimation (50% yield); m.p. 95–97°C.

4.4. Preparation of monoarylhydrazones: general procedure

A mixture of the appropriate arylaldehyde (10.8 mmol) and 100% hydrazine hydrate (2.1 ml, 43 mmol) in 15 ml of *i*-PrOH was heated under reflux until the solution was completely homogeneous. The product crystallized after cooling the reaction mixture. 4-Chlorobenzaldehyde monohydrazone was extracted with benzene after removing the solvent reaction, then benzene was vacuum evaporated. The other products were filtered off, washed with *i*-PrOH and dried. All these products were used in the next step without further purification. Ar, (%), m.p.: 4-ClC_6H_4, (83%), viscous pale yellow semi-solid; 4-$NO_2C_6H_4$, (66%), 130–132°C (Lit. [15] 134°C); 1-naph, (48%), 87–90°C; 2-naph, (80%), 149–150°C and 6-methoxy-2-naph, (92%), 169–172°C.

4.5. 2-Arylmethylene-N-arylhydrazinecarboxamides

*4.5.1. Compounds **1–12**: general procedure — Method A*

A mixture of the appropriate arylhydrazinecarboxamide (6.6 mmol) and the corresponding substituted aromatic aldehyde (6.0 mmol) in 15 ml ethanol was stirred and heated under reflux for 1–2 h and the precipitate was collected and recrystallized from a suit-

able solvent (Table 1). In order to obtain compounds **4** and **5** a catalytic amount of acetic acid was necessary.

^{1}H NMR and IR spectral data of representative compounds are as follows:

Compound **6**. ^{1}H NMR δ (ppm): 10.84 (s, 1H, CH), 9.19 (s, 1H, NHCO), 8.04–7.42 (m, 13H, arom. and CONH). IR ν (cm^{-1}): 1700, 1580, 1400, 1300, 820.

Compound **11**. ^{1}H NMR δ (ppm): 10.85 (s, 1H, CH), 9.15 (s, 1H, NHCO), 8.05–7.45 (m, 12H, arom. and CONH). IR ν (cm^{-1}): 1720, 1700, 1470, 1300, 820, 710.

*4.5.2. Compounds **13–19**: general procedure — Method B*

To a solution of the desired monoarylhydrazone (2.5 mmol) in benzene with stirring at r.t., was added dropwise a solution of arylisocyanate (3.0 mmol) in benzene. The product that precipitated was filtered off and recrystallized from a suitable solvent (Table 1).

The ^{1}H NMR and IR spectral data of representative compounds are as follows:

Compound **12**. ^{1}H NMR δ (ppm): 10.70 (s, 1H, CH), 9.13 (s, 1H, NHCO), 7.98–6.99 (m, 12H, arom. and CONH), 3.81 (s, 3H, OCH_3). IR ν (cm^{-1}): 1690, 1530, 1240, 820.

Compound **19**. ^{1}H NMR δ (ppm): 11.04 (s, 1H, CH), 9.54 (s, 1H, NHCO), 8.25–7.24 (m, 11H, arom. and CONH), 3.91 (s, 3H, OCH_3). IR ν (cm^{-1}): 1700, 1600, 1510, 1330, 1220, 850.

*4.6. 2-(4-Chlorophenylmethyl)-N-(1-naphthyl)hydrazinecarboxamide (**20**)*

4-Chlorobenzaldehyde monohydrazone (1.5 g) was dissolved in 30 ml EtOH and 5 mg of 10% Pd/C were added as catalyst. Then the mixture was hydrogenated for 3 h at 30 psi. The catalyst was filtered off and the solvent was vacuum evaporated; 1.0 g of 4-chlorobenzylhydrazine was obtained (66% yield, m.p. <50°C), which was dissolved in 10 ml benzene and then a solution of 0.9 ml of commercial 1-naphthylisocyanate in 3 ml benzene was added dropwise at r.t. The white product that immediately precipitated was collected and washed with the same solvent. Then it was recrystallized from EtOH (39% yield, m.p. 220–222°C). ^{1}H NMR δ (ppm): 9.20–9.08 (m, 2H, NHNH), 8.31–7.30 (m, 12H, arom. and CONH), 4.07–3.99 (m, 2H, CH_2). IR ν (cm^{-1}): 1680, 1560, 1460, 760.

*4.7. 2-(4-Chlorophenylmethylene)-N-(1-naphthylmethyl)hydrazinecarboxamide (**21**)*

N-(1-Naphthylmethyl)hydrazinecarboxamide was synthesized according to the method described above for preparing *N*-phenylhydrazinecarboxamide, from *N*-(1-naphthylmethyl)urea and hydrazine hydrate, then *N*-(1-naphthylmethyl)hydrazinecarboxamide chlorohydrate was prepared (40% yield, m.p. 155–160°C) and 0.60 g (2.4 mmol) of this compound was dissolved in 20 ml water. To this solution 0.39 g (4.2 mmol) of sodium acetate was added. Then a solution of 0.67 g (4.8 mmol) of 4-chlorobenzaldehyde in 5 ml EtOH was added dropwise at r.t. Immediately a white solid precipitated. The mixture reaction was heated under reflux for 1 h to complete the reaction. The product was filtered off and washed with EtOH, the crude was pure enough (90% yield, m.p. 225–228°C). ^{1}H NMR δ (ppm): 10.31 (s, 1H, CH), 8.72 (s, 1H, NHCO), 7.93–7.41 (m, 12H, arom. and CONH), 6.53 (s, 2H, CH_2). IR ν (cm^{-1}): 1680, 1520, 1460, 820.

4.8. Binding to DNA

DNA solution: Calf thymus DNA (12.5 mg) was slowly magnetically stirred in 5 ml Tris–HCl buffer (10 mM, pH 7.4) for 24 h at 4°C. From this solution, 0.6 ml were taken off and diluted with the same buffer to 25 ml.

Test compound solution: it was prepared at 10^{-4} M concentration using a minimal ethanol volume and water, then it was diluted to a concentration of 2×10^{-5} M. A 3 ml sample of this resulting solution was mixed with 3 ml of DNA solution described above and, after slowly rotating the mixture for 24 h, its UV spectra were recorded using a 1 cm cell at 20°C.

4.9. Cyclic voltammetry

Potentiometer: EQMAT-S1; glassy carbon working electrode, calomel reference electrode, gold wire antielectrode.

Supporting electrolyte: tetrabutylammonium perchlorate (TBAP) 50 mM; sample concentration: 0.5 mM. Sensibility: 25 μA/div. Solvent: acetonitrile of electrochemical purity. Inert bubbling gas: nitrogen. Sweep rate: 50 mV/s. Potential scanning: between $+2000$ and -2000 mV.

4.10. Topoisomerase I inhibition assay

Inhibition of topoisomerase I activity was determined using a relaxation assay described [16] with modifications. Plasmid pHOT1 (Topogen, USA) was used as a substrate in a final reaction volume of 20 μl containing 10 mM Tris–HCl, 1 mM EDTA, 0.15 M NaCl, 0.1% BSA, 0.1 mM spermidine, 5% glycerol and 2 units of purified human topoisomerase I (Topogen, USA). The drug was pre-dissolved in DMSO and tested at doses of 100, 10 and 1 μg/ml. The reaction was carried out at 37°C for 30 min and terminated by addition of 10% SDS and proteinase K, 50 μg/ml (Sigma, USA). After

digestion at 37°C for 30 min, 0.025% of loading dye was added to the reaction medium. The sample, previously extracted with chloroform: isoamyl alcohol (CIA), was submitted to a 1% agarose gel electrophoresis using TAE buffer for 4 h at 36 V at r.t. For the quantitative determination of topo I activity, photographic negatives were scanned (Hewlett Packard Scanjet 11p) and the bands analyzed with the Jandel SigmaScan image measurement analyzing program. The area representing supercoiled DNA, migrating as bands at the bottom of the gel, was determined.

Drug-induced inhibition of topoisomerase I-mediated DNA relaxation was computed from Eq. (2):

$$\% \text{ Inhibition: } (SC_{ED}/SC_{O}) \times 100 \qquad (2)$$

where SC_{ED} represents the fraction of supercoiled DNA measured in the presence of enzyme and drug and SC_{O} represents the untreated supercoiled DNA. IC_{50} values were calculated using the Finney probit analysis computer program [17].

Acknowledgements

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