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Original article

Synthesis and cytotoxic activity of substituted 7-aryliminomethyl derivatives of camptothecin

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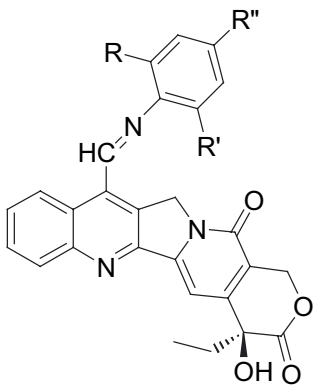
Abstract

A series of imines derived from camptothecin-7-aldehyde (CPT-CHO) and aromatic amines were synthesised and tested for their cytotoxicity against tumour cell line H460, that expresses a high level of topoisomerase I. In general *ortho*-substituted compounds showed higher cytotoxic potency than the corresponding *para*-substituted imines. This effect was dependent on the nature of the substituent. Structure–activity relationships were studied by calculation of docking energy with a model of the ternary complex camptothecin–DNA–topoisomerase I. The ability of selected compounds to stimulate the topoisomerase I-mediated DNA cleavage and the persistence of the cleavable complex were consistent with the cytotoxic activity.

Keywords: Camptothecin; Imines; Antitumour; Topoisomerase

1. Introduction

Derivatives of the natural alkaloid camptothecin (CPT) are an emerging class of effective anticancer agents [1].



The recent advances in molecular and cellular pharmacology of these agents and the clinical success of topotecan (Hycamtin®) and irinotecan (Camptosar®) [2] have stimulated extensive efforts for the discovery of analogues with an improved pharmacological profile and/or enhanced efficacy against human tumours.

In our previous papers [3,4] we have stressed the importance of lipophilic groups linked to position 7 of camptothecin to obtain potent cytotoxic activity. In particular we have prepared a series of iminomethyl substituted camptothecins via imination of the easily available camptothecin-7-aldehyde (CPT-CHO) [3]. Among these derivatives the compound obtained by condensation of CPT-CHO with aniline exhibited a potent cytotoxic activity against the H460 tumour cell line (IC_{50} = 0.13 μM).

In order to investigate the influence of the aromatic ring on the activity of this class of compounds, we planned to introduce various substituents in different positions of the ring itself. We report here on the synthesis and the pattern of cytotoxic response of a series of *ortho*- and *para*-substituted 20*S*-7-aryliminomethyl derivatives of camptothecin. One of the most potent compounds of this series was studied in a gastric carcinoma xenograft growing in athymic nude mice, and other selected compounds were tested for their ability to stabilise the topoisomerase I–DNA cleavable complex.

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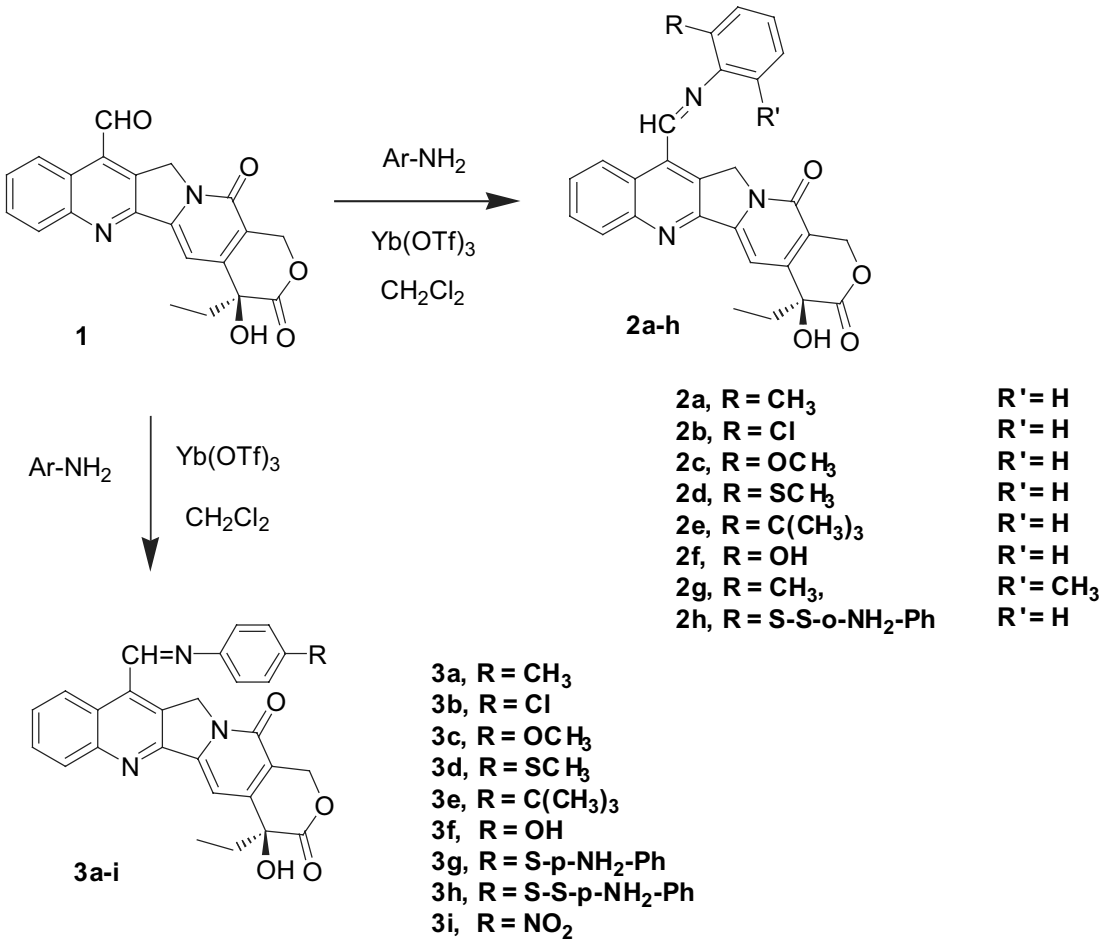


Scheme 1.

Structure–activity relationships of this new series of camptothecin derivatives were studied by calculating their interaction with the recently reported X-ray crystal structure of human topoisomerase I (Topo I) covalently joined to a double-stranded DNA and bound to topotecan [5].

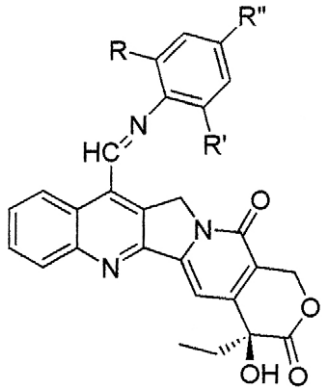
2. Results and discussion

The 7-iminoderivatives were obtained in good yield from 20*S*-CPT-CHO (**1**), on its turn prepared according to the procedure described by Sawada et al. [6] by the $Yb(TfO)_3$-catalysed condensation reported by Kobayashi et al. [7] (Scheme 1).

The compounds prepared were evaluated for their cytotoxicity against the human non-small lung carcinoma cell line H460, using topotecan as a reference compound. This cell model was chosen for its sensitivity to topoisomerase I inhibitors, likely related to overexpression of the target enzyme [8]. The cellular pharmacology study shows that all the imines prepared exhibited a potent cytotoxic activity, superior to that of topotecan under the same conditions (Table 1).

From the cytotoxicity values (IC_{50}) it emerges that the presence of substituents in *ortho* position results in a substantial increase of the cytotoxic activity compared to the unsubstituted derivative (IC_{50} ranging from 0.03 to 0.11 μM) independently from the electronic and steric characteristics of the groups. On the contrary the introduction of substituents in *para* position is more dependent on the nature of the residues and usually these derivatives are less potent than the corresponding *ortho* ones. The most potent compound of these series, **2f**, with an OH-substituent is about seven times more potent than the corresponding *para* analogue. This difference could be ascribed to a combination of factors, like intracellular drug accumulation (which may depend on solubility, stability, non-specific binding to serum, and other factors) or by a different interaction at the cleavage site. It could also be hypothesised that the activity of these imines is due to the liberation of CPT-CHO and that the measured difference in activity depends on a lower rate of hydrolysis of the *ortho*-substituted derivatives because of steric hindrance. Apart from the observation that CPT-CHO is much less active (IC_{50} = 0.39 μM) [9] against this hypothesis is the fact, reported in our previous paper [3], that the corresponding amines, compounds not hydrolysable, show an activity very similar to that of the imines. To investigate the relevance of the binding of the drug to the Topo I covalent complex with DNA, we compared the intermolecular interaction energy of the couples of *ortho*- and *para*-substituted compounds with the model of topoisomerase I cleavage site. The starting

Table 1
In vitro cytotoxic activity of CPT derivatives on H460 cell line



Compound	R	R′	R″	IC_{50} (μM) H460
Topotecan				1.38 ± 0.95
2a	CH_3	H	H	0.11 ± 0.04
2b	Cl	H	H	0.075 ± 0.02
2c	OCH_3	H	H	0.058 ± 0.025
2d	SCH_3	H	H	0.066 ± 0.006
2e	$C(CH_3)_3$	H	H	0.065 ± 0.006
2f	OH	H	H	0.032 ± 0.003
2g	CH_3	CH_3	H	0.156 ± 0.042
2h	S-S-*o*-NH_2-Ph	H	H	0.049 ± 0.011
3a	H	H	CH_3	0.18 ± 0.003
3b	H	H	Cl	0.086 ± 0.002
3c	H	H	OCH_3	0.166 ± 0.004
3d	H	H	SCH_3	0.074 ± 0.01
3e	H	H	$C(CH_3)_3$	0.09 ± 0.01
3f	H	H	OH	0.22 ± 0.076
3g	H	H	S-*p*-NH_2-Ph	0.24 ± 0.069
3h	H	H	S-S-*p*-NH_2-Ph	0.387 ± 0.04
3I	H	H	NO_2	0.28 ± 0.06

model of the enzyme (Topo I model) was obtained from the X-ray crystal structure of human topoisomerase I covalently joined to a double-stranded DNA [4] and bound to topotecan. Not the whole structure was used, but a smaller one, mimicking the intercalation binding pocket and its surroundings, formed by three base pairs upstream and downstream of the cleavage site. A certain number of amino acids were chosen including the one covalently bonded to the DNA (Tyr-723), those indicated in the literature as CPT resistance point mutations in human Topo I (Asp-533 [10], Arg-364 [11], Asn-722 [12], Phe-361 [13] and Gly-363 [14]), and some more to fill the area around the cleavage site. The final model of Topo I considered consists of six base pairs (5′ CTT-Tyr-723, GGA 3′; 3′ GAACCT 5′) and 70 amino acids[1]. The imines were inserted in the model, one by one, superimposing the part of the molecule in common with topotecan, and the complexes obtained minimised as described. Fig. 1 shows all the base pairs and some amino acids, as obtained from Ref. [5] and used as starting model of the cleavage site, together with compound **2b**.

The binding affinity of the appropriate imine with the model of Topo I was evaluated by calculating the intermolecular interaction energy between the imine and the Topo I model (Docking module in Insight II). Interestingly (Table 2) in five out of six cases, the total interaction energy resulted lower (better docking) for the *ortho*-substituted compound, in accordance with the trend of cytotoxic activity.

The hypothesis that the increased potency of the *ortho*-substituted derivatives is mainly due to a stronger interaction at the cleavage site is also supported by the results obtained from experiments of topoisomerase I-mediated DNA cleavage. We have investigated the ability of some selected compounds to stimulate the cleavage, using the purified human enzyme. As shown in Fig. 2, the two most active *ortho*- and also two of the *para* derivatives are very potent as topoisomerase I poisons and more effective than SN38 (the active principle of irinotecan), used as reference compound, in the stabilisation of the ternary cleavable complex. In particular it is interesting to notice that the cleavage persistence is around 80% after 10 min for the *ortho*-hydroxy derivative (**2f**) while the corresponding *para*-substituted compound (**3f**) shows a significantly lower percentage of persistence after the same period of time.

3. Conclusions

As already observed for 7-oxyiminomethyl derivatives [2], 7-aryliminomethyl derivatives of camptothecin exhibit a high cytotoxic potency. This finding implies that substituents at the seven positions provide a favourable interaction of the drug in the ternary drug–DNA–enzyme complex, as supported by persistence of the DNA cleavage (Fig. 2), and by the docking calculations. This hypothesis is also supported by the observed modulation of cytotoxic activity in dependence on the nature and position of the substituents in compounds of this series. In vivo antitumour activity studies with **2h** suggest the interest of the novel camptothecin derivatives in terms of efficacy and tolerability. Indeed, this derivative was significantly effective in the treatment of a human gastric carcinoma model (MKN28), because the oral administration at well tolerated doses (4 mg kg^{-1}, with a q4dx4 schedule) produced 70% inhibition of tumour growth without manifestations of toxicity.

4. Experimental

All reagents and solvents were reagent grade or were purified by standard methods before use. Melting points

[1] The 70 amino acids are: Val-226, Tyr-338, Gly-339, Arg-349, Ile-350, Ala-351, Asn-352, Phe-353, Lys-354, Ile-355, Glu-356, Phe-361, Arg-362, Gly-363, Arg-364, Lys-374, Trp-412, Leu-413, Val-414, Ser-415, Trp-416, Lys-425, Tyr-426, Ile-427, Met-428, Leu-429, Asn-430, Pro-431, Ser-432, Ser-433, Lys-436, Gly-437, Glu-438, Arg-488, Ala-489, Lys-532, Asp-533, Ser-534, Ile-535, Arg-536, Tyr-537, Arg-590, Thr-591, Tyr-592, Leu-629, Cys-630, Asn-631, His-632, Gln-633, Arg-634, Ala-635, Pro-636, Pro-637, Lys-712, Gln-713, Ile-714, Ala-715, Gly-717, Thr-718, Ser-719, Lys-720, Leu-721, Asn-722, Tyr-723, Gln-748, Arg-749, Glu-750, Lys-751, Phe-752, Ala-753.

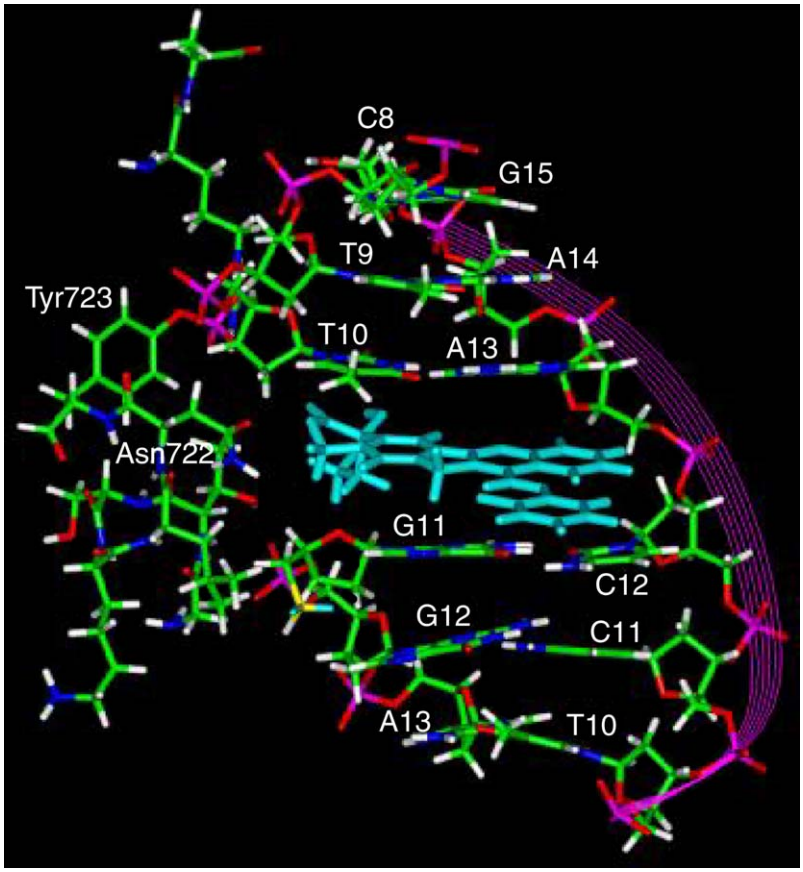


Fig. 1. Base pairs and some amino acids used as starting model of the cleavage site, together with compound **2b**.

(m.p.) were determined in open capillaries on a Büchi melting point apparatus and are uncorrected. Column chromatography was carried out on flash silica gel (Merck 230–400 mesh). TLC analysis was conducted on silica gel plates (Merck $60F_{254}$). NMR spectra were recorded in DMSO-d_6 (when not otherwise stated) at 300 MHz with a Bruker instrument. Chemical shifts (δ values) and coupling constants (J values) are given in ppm and Hz, respectively. Mass spectra were recorded at an ionising voltage of 70 eV on a Finnigan TQ70 spectrometer. The relative intensities of mass spectrum peaks are listed in parentheses. Solvents were routinely distilled prior to use; anhydrous tetrahydrofuran (THF) and ether (Et_2O) were obtained by distillation from sodium-benzophenone ketyl; dry methylene chloride was obtained by distillation from phosphorus pentoxide. All reactions requiring anhydrous conditions were performed under a positive nitrogen flow, and all glassware was oven-dried and/or flame dried. Analyses indicated by the symbols of the elements or functions were within ±4% of the theoretical values.

Table 2
Ligand–Topo I–DNA interaction energies (kcal mol^{-1})

Compound	Interaction energy (kcal mol^{-1})
2a	–56.13
3a	–55.82
2b	–55.57
3b	–57.25
2c	–53.62
3c	–52.80
2d	–52.93
3d	–50.66
2e	–61.49
3e	–54.63
2f	–58.90
3f	–56.49

4.1. Synthesis of compounds: general procedure

To a suspension of 20*S*-CPT-CHO (**1**) (100 mg, 0.26 mmol) in 7 ml anhydrous CH_2Cl_2, the appropriate amine (0.78 mmol) and $Yb(OTf)_3$ (16 mg, 0.03 mmol) were added. The resulting mixture was stirred at room temperature until the reaction was complete. After filtering the sieves the solvent was evaporated, and the product purified by flash chromatography on silica gel (Merck 230–400 mesh).

2a. The solution is stirred for 24 h. Flash chromatography (eluent: CH_2Cl_2/MeOH 95:5). Yellow powder. Yield 55%, m.p. 247–248 °C dec., ^{1}H NMR (DMSO-d_6) δ: 0.87 (t, J = 7 Hz, H_3-18) 1.7–1.9 (m, H_2-19) 2.5 (s, Ar–CH_3) 5.4 (s, H_2-17) 5.60 (s, H_2-5) 6.55 (s, –OH) 7.25–7.50 (m, 4H Ar, H-14) 7.75 (m, H-11) 7.95 (m, H-10) 8.25 (dd, H-12) 9.10 (dd, H-9) 9.65 (s, CH=N).

2b. The solution is stirred for 48 h. Flash chromatography (eluent: CH_2Cl_2/MeOH 98:2). Yellow powder. Yield 27%, m.p. >240 °C dec., ^{1}H NMR (DMSO-d_6) δ: 0.83 (t, J = 7 Hz,

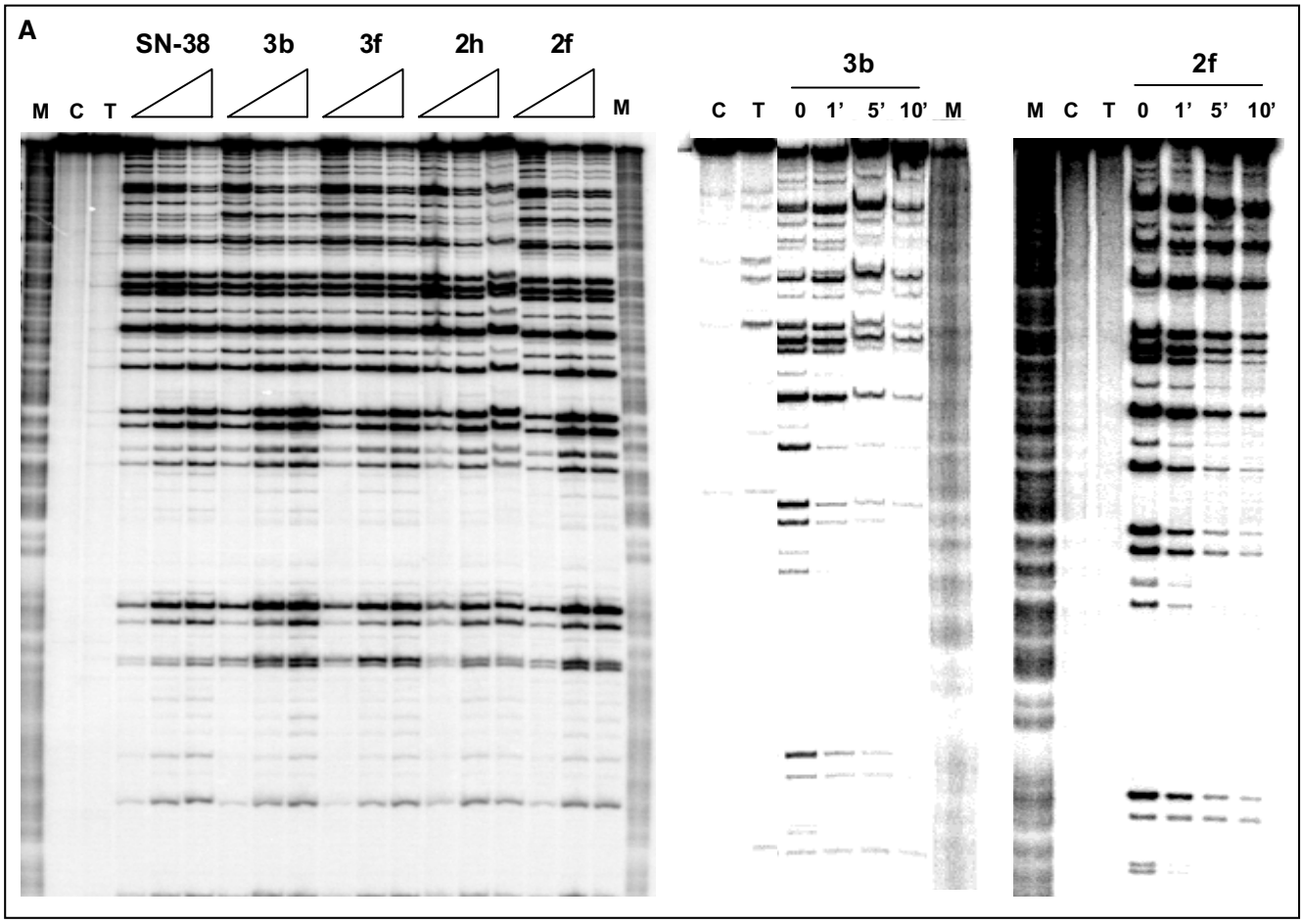


Fig. 2. Stimulation and persistence of topoisomerase I-mediated DNA cleavage in the presence of 7-modified camptothecins. (A) Dose dependent stimulation and persistence of DNA cleavage. The samples were reacted for 30 min with 1, 10 and 50 μM drug. The triangle indicates the increase of drug concentration. For the study of cleavage persistence the samples were reacted with 10 μM drug. DNA cleavage was then reversed at the indicated time by adding 0.6 M NaCl. The 100% value is referred to the extent of DNA cleavage at 30 min of incubation. (B) Densitometric analysis of the persistence of topoisomerase I-mediated DNA cleavage. (C) Control DNA (751-bp); T, reaction without drug; M, purine markers.

H_3-18) 1.7–1.9 (m, H_2-19) 5.45 (s, H_2-17) 5.60 (s, H_2-5) 6.50 (s, –OH) 7.35–7.50 (m, H-14; 4H Ar) 7.85 (m, H-11) 7.95 (m, H-10) 8.30 (dd, H-12) 9.10 (dd, H-9) 9.70 (s, CH=N).

2c. The solution is stirred for 30 h. Flash chromatography (eluent: CH_2Cl_2/MeOH 98:2). Yellow powder. Yield 24%, m.p. 244–246 °C dec., ^{1}H NMR (DMSO-d_6) δ: 0.83 (t, J = 7 Hz, H_3-18) 1.7–1.9 (m, H_2-19) 3.95 (s, OCH_3) 5.45 (s, H_2-17) 5.55 (s, H_2-5) 6.45 (s, –OH) 7.0–7.50 (m, H-14; 4H Ar) 7.7 (m, H-11) 7.85 (m, H-10) 8.25 (dd, H-12) 8.9 (dd, H-9) 9.70 (s, CH=N).

2d. The solution is stirred for 48 h. Flash chromatography (eluent: CH_2Cl_2/MeOH 99:1). Yellow powder. Yield 9%, m.p. >250 °C dec., ^{1}H NMR (DMSO-d_6) δ: 0.83 (t, J = 7 Hz, H_3-18) 1.7–1.9 (m, H_2-19) 2.50 (s, SCH_3) 5.40 (s, H_2-17) 5.70 (s, H_2-5) 6.45 (s, –OH) 7.25–7.35 (m, H-14; 3H Ar) 7.6 (m, 1H Ar) 7.8 (m, H-11) 7.95 (m, H-10) 8.30 (dd, H-12) 9.10 (dd, H-9) 9.55 (s, CH=N).

2e. The solution is stirred for 16 h. Flash chromatography (eluent: CH_2Cl_2/MeOH 99:1). Yellow powder. Yield 27%, m.p. 215 °C dec., ^{1}H NMR (DMSO-d_6) δ: 0.85 (t, J = 7 Hz, H_3-18) 1.45 (s, 9H, *t*But) 1.7–1.9 (m, H_2-19) 5.35–5.75 (m, H_2-17, H_2-5) 6.50 (s, –OH) 7.05–7.5 (m, H-14; 4H Ar) 7.75–7.85 (m, H-11) 7.88–7.95 (m, H-10) 8.25 (dd, H-12) 8.95 (dd, H-9) 9.45 (s, CH=N).

2f. The solution is stirred for 48 h. Flash chromatography (eluent: CH_2Cl_2/MeOH 98:2). Yellow powder. Yield 42%, m.p. 252–254 °C dec., ^{1}H NMR (DMSO-d_6) δ: 0.87 (t, J = 7 Hz, H_3-18) 1.7–1.9 (m, H_2-19) 5.4 (s, H_2-17) 5.60 (s, H_2-5) 6.55 (s, –OH) 6.90–7.5 (m, 4H Ar, H-14) 7.85–8.0 (m, H-11, H-10) 8.35 (dd, H-12) 8.90 (dd, H-9) 9.70 (s, CH=N).

2g. The solution is stirred for 48 h. Flash chromatography (eluent: CH_2Cl_2/MeOH 95:5). Yellow powder. Yield 50%, m.p. 250 °C dec., ^{1}H NMR (DMSO-d_6) δ: 0.87 (t, J = 7 Hz, H_3-18) 1.7–1.9 (m, H_2-19) 2.25 (s, 2Ar–CH_3) 5.4 (s, H_2-17) 5.60 (s, H_2-5) 6.55 (s, –OH) 7.0–7.30 (m, 3H Ar) 7.40 (s, H-14) 7.8 (m, H-11) 7.9 (m, H-10) 8.25 (dd, H-12) 8.85 (dd, H-9) 9.5 (s, CH–N).

2h. The solution is stirred for 24 h. Flash chromatography (eluent: CH_2Cl_2/MeOH 98:2). Yellow powder. Yield 45%, ^{1}H NMR (DMSO-d_6) δ: 0.83 (t, J = 7 Hz, H_3-18) 1.7–1.9 (m, H_2-19) 5.35–5.75 (6H, m, H_2-5 + H-17 + NH_2), 6.40 (1H, m, ArH), 6.5–6.6 (2H, m, 1ArH + OH), 6.90 (1H, m, Ar), 7.25–7.45 (4H, m, 3Ar + H-14), 7.15–8.0 (4H, m, 4Ar), 8.25 (1H, dd), 9.75 (1H, s, CH=N).

3a. The solution is stirred for 24 h. Flash chromatography (eluent: CH_2Cl_2/MeOH 99:1). Yellow powder. Yield 27%, m.p. 159–160 °C dec., ^{1}H NMR (DMSO-d_6) δ: 0.83 (t, J = 7 Hz, H_3-18) 1.7–1.9 (m, H_2-19) 2.35 (s, Ar–CH_3) 5.37 (s, H_2-17) 5.5 (s, H_2-5) 6.45 (s, –OH) 7.25–7.35 (m, H-14; 2H Ar) 7.4–7.5 (m, 2H Ar) 7.7 (m, H-11) 7.85 (m, H-10) 8.16 (dd, H-12) 8.9 (dd, H-9) 9.55 (s, CH–N).

3b. The solution is stirred for 15 h. Flash chromatography (eluent: CH_2Cl_2/MeOH 98:2). Yellow powder. Yield 31%, m.p. 246–247 °C dec., ^{1}H NMR (DMSO-d_6) δ: 0.83 (t, J = 7 Hz, H_3-18) 1.7–1.9 (m, H_2-19) 5.40 (s, H_2-17) 5.55 (s, H_2-5) 6.45 (s, –OH) 7.35 (s, H-14) 7.50–7.60 (m, 4H Ar) 7.85 (m, H-11) 7.95 (m, H-10) 8.25 (dd, H-12) 8.95 (dd, H-9) 9.55 (s, CH=N).

3c. The solution is stirred for 48 h. Flash chromatography (eluent: CH_2Cl_2/MeOH 98:2). Yellow powder. Yield 76%, m.p. 252–255 °C dec., ^{1}H NMR (DMSO-d_6) δ: 0.87 (t, J = 7 Hz, H_3-18) 1.7–1.9 (m, H_2-19) 3.8 (s, –OCH_3) 5.4 (s, H_2-17) 5.45 (s, H_2-5) 6.55 (s, –OH) 7.05 (d, 2H Ar) 7.35 (s, H-14) 7.60 (d, 2H Ar) 7.85 (m, H-11) 7.9 (m, H-10) 8.25 (dd, H-12) 8.8 (dd, H-9) 9.5 (s, CH=N).

3d. The solution is stirred for 22 h. Flash chromatography (eluent: CH_2Cl_2/MeOH 98:2). Yellow powder. Yield 36%, m.p. 160 °C dec., ^{1}H NMR (DMSO-d_6) δ: 0.87 (t, J = 7 Hz, H_3-18) 1.7–1.9 (m, H_2-19) 2.55 (s, –SCH_3) 5.45 (s, H_2-17) 5.55 (s, H_2-5) 6.50 (s, –OH) 7.35 (s, H-14) 7.40 (d, 2H Ar) 7.55 (d, 2H Ar) 7.80 (m, H-11) 7.9 (m, H-10) 8.20 (dd, H-12) 8.95 (dd, H-9) 9.7 (s, CH=N).

3e. The solution is stirred for 1.5 h. Flash chromatography (eluent: CH_2Cl_2/MeOH 99:1). Yellow powder. Yield 50%, m.p. 250 °C dec., ^{1}H NMR (DMSO-d_6) δ: 0.88 (t, J = 7 Hz, H_3-18) 1.30 (s, *t*But) 1.75–1.95 (m, H_2-19) 5.45 (s, H_2-17) 5.55 (s, H_2-5) 6.55 (s, –OH) 7.35 (s, H-14) 7.45–7.60 (m, 4H Ar) 7.80 (m, H-11) 7.95 (m, H-10) 8.25 (dd, H-12) 8.95 (dd, H-9) 9.7 (s, CH=N).

3f. The solution is stirred for 3 h. Flash chromatography (eluent: CH_2Cl_2/MeOH 96:4). Yellow powder. Yield 79%, m.p. 250 °C dec., ^{1}H NMR (DMSO-d_6) δ: 0.90 (t, J = 7 Hz, H_3-18) 1.75–2.0 (m, H_2-19) 5.4 (s, H_2-17) 5.55 (s, H_2-5) 6.50 (s, –OH) 6.90 (d, 2H Ar) 7.35 (s, H-14) 7.55 (d, 2H Ar) 7.80 (m, H-11) 7.90 (m, H-10) 8.25 (dd, H-12) 9.0 (dd, H-9) 9.70 (s, CH=N).

3g. The solution is stirred for 2 days. Flash chromatography (eluent: CH_2Cl_2/MeOH 99:1).

Yellow powder. Yield 8%, m.p. 187–188 °C dec., ^{1}H NMR (DMSO-d_6) δ: 0.87 (t, J = 7 Hz, H_3-18) 1.7–1.9 (m, H_2-19) 5.4 (s, H_2-17) 5.55 (s, H_2-5 + NH_2) 6.55 (s, –OH) 6.65 (m, 2H Ar) 7.10–7.50 (m, 6H Ar + H-14) 7.8 (m, H-11) 7.9 (m, H-10) 8.30 (dd, H-12) 9.0 (dd, H-9) 9.5 (s, CH=N).

3h. The solution is stirred for 44 h. Flash chromatography (eluent: CH_2Cl_2/MeOH 99:1). Orange powder. Yield 14%, m.p. 154–155 °C dec., ^{1}H NMR (DMSO-d_6) δ: 0.83 (t, J = 7 Hz, H_3-18) 1.7–1.9 (m, H_2-19) 5.40 (s, H_2-17) 5.55 (s, H_2-5 + NH_2) 6.50 (s, –OH) 6.55 (m, 2H Ar) 7.25 (m, 2H Ar) 7.35 (s, H-14) 7.60 (m, 4H Ar) 7.8 (m, H-11) 7.9 (m, H-10) 8.25 (dd, H-12) 9.0 (dd, H-9) 9.70 (s, CH=N).

3i. The solution is stirred for 48 h. Flash chromatography (eluent: CH_2Cl_2/MeOH 98:2). Orange powder. Yield 32%, m.p. 260–265 °C dec. ^{1}H NMR (DMSO-d_6) δ: 0.85 (t, J = 7 Hz, H_3-18) 1.7–1.9 (m, H_2-19) 5.35 (s, H_2-17) 5.48 (s, H_2-5) 6.45 (s, –OH) 7.3 (s, H-14) 7.6–7.7 (m, 2Ar) 7.8 (m, H-11) 7.9 (m, H-10) 8.25 (dd, H-12) 8.35–8.40 (m, 2Ar) 8.9 (dd, H-9) 9.67 (s, CH=N).

5. Assessment of antitumour activity

Cells were cultured in RPMI-1640 containing 10% foetal calf serum. Cytotoxicity was assessed by growth inhibition

assay after 1 h drug exposure. Cells in the logarithmic phase of growth were harvested and seeded in duplicates into six-well plates. Twenty-four hours after seeding, cells were exposed to the drug and harvested 72 h after exposure and counted with a Coulter counter. IC_{50} is defined as the inhibitory drug concentration causing a 50% decrease of cell growth over that of untreated control. All compounds were dissolved in DMSO prior to dilution into the biological assay.

6. Topoisomerase I-dependent DNA cleavage assay

A gel purified 751-bp BamHI-EcoRI fragment of SV40 DNA was used for the cleavage assay. DNA fragments were uniquely 3′-end labelled. Topoisomerase I DNA cleavage reactions (20 000 cpm per sample) were performed in 20 μl of 10 mM Tris–HCl (pH 7.6), 150 mM KCl, 5 mM $MgCl_2$, 15 μg ml^{-1} BSA, 0.1 mM dithiotreitol, and the human recombinant enzyme (full length topoisomerase I) [15] for 30 min at 37 °C. Reactions were stopped by 0.5% SDS and 0.3 mg ml^{-1} of proteinase K for 45 min at 42 °C. Persistence of DNA cleavage at different time points was examined by adding 0.6 M NaCl after 30 min of incubation. After precipitation DNA was resuspended in denaturing buffer (80% formamide, 10 mM NaOH, 0.01 M EDTA and 1 mg ml^{-1} dyes) before loading on a denaturing 7% polyacrylamide gel in TBE buffer. Overall DNA cleavage levels were measured with a Phospholmager 425 model (Molecular Dynamics).

7. Molecular modelling

Three-dimensional molecular models of the new imines were built on a Silicon Graphics O2, using the programs Insight II and Discover (Accelrys Inc., San Diego, CA). Minimisations were performed with the AMBER all-atom forcefield [16] and the conjugate gradients algorithm. For atomic partial charges of the ligand atoms we used Mulliken charges calculated on the minimised structures using the MOPAC program [17] with the MNDO Hamiltonian.

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