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Pyranonaphthoquinone derivatives of eleutherin, ventiloquinone L, thysanone and nanaomycin A possessing a diverse topoisomerase II inhibition and cytotoxicity spectrum

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ABSTRACT

A series of pyranonaphthoquinone derivatives related to the known topoisomerase II inhibitor eleutherin **1** have been shown to act as specific topoisomerase II catalytic inhibitors, with several analogues displaying greater potency than the natural product itself. Amongst the compounds tested were the natural products ventiloquinone L **4** and thysanone **8** with a diverse range of topoisomerase II inhibition properties being observed. Interestingly, the natural products are generally weaker inhibitors than their synthetic counterparts, emphasising that subtle changes in the basic molecular structure of a natural product led to significant changes in the inhibition profile. It has also been demonstrated for the first time that analogues related to nanaomycin A and cardinalin-type dimeric pyranonaphthoquinones exhibit potent topoisomerase II inhibitory properties. With respect to structural features, it appears that the nature of the substituents at C1 on the pyran ring and oxygenated substituents on the aryl ring are critical for anti-topoII activity.

Importantly, the topoisomerase II inhibition strength does not correlate well with the measured cytotoxicity against yeast, indicating that other molecular features in the pyranonaphthoquinone family must be considered for the design and use of this structural class as highly specific topoisomerase II inhibitors.

1. Introduction

Topoisomerases are molecular targets accounting for about 50% of the current targets for chemotherapy.[1] In all living beings, these enzymes catalyse changes in the topology of the DNA molecule which facilitate important physiological events within the cell such as transcription, replication and chromosome segregation.[2]

Two types of topoisomerases can be differentiated on the basis of their mechanism of action. Type I topoisomerases catalyse the relaxation of positive supercoiling by making single-strand breaks in one of the DNA strands.[3] Type II topoisomerases make transient double strand breaks allowing another DNA molecule to pass through it. Type II topoisomerases can both relax supercoiled DNA and unknot two or more DNA molecules in a process known as decatenation. Decatenation is essential in order to allow a faithful segregation of the replicated genetic material to the daughter cells during cell division.[3,4]

Topoisomerases are good antitumour targets firstly, because they are highly active in cells which are proliferating[5,6] and secondly, they generate transient strand breaks and therefore topoisomerases are potential cytotoxic molecular scissors. In fact, most of the known antitumour drugs that target topoisomerases act by enhancing such scissor activities.[7–10] These drugs, known as topoisomerase poisons, generate DNA damage[1,8] which is the ultimate cause of induced cell death for most cancer cells. Examples of such poisons can be found for both topoisomerases. The camptothecins are specific TopoI poisons,[11] whereas epipodophyllotoxins (etoposide), aminoacridines (amsacrine) and anthracyclines (doxorubicin, daunorubicin, etc.) are potent TopoII poisons.[10,12,13] Aside from poisons, other groups of drugs with potent antitumour activity have been shown to purely act as catalytic inhibitors of the enzymes. This is the case for the anti-TopoII agents merbarone and the family of bisdioxopiperazines.[14,15] There is an increasing interest in this group of drugs as resistance to topoisomerase poisons based on mutations of the TopoII enzyme is a common pattern for failure of chemotherapy.[16] However, such mutations may also reduce overall enzymatic activity thus rendering tumours hypersensitive to catalytic inhibition of the enzyme.

Interestingly, the quinone moiety has been shown to exhibit multiple anti-proliferative activity against a broad spectrum of

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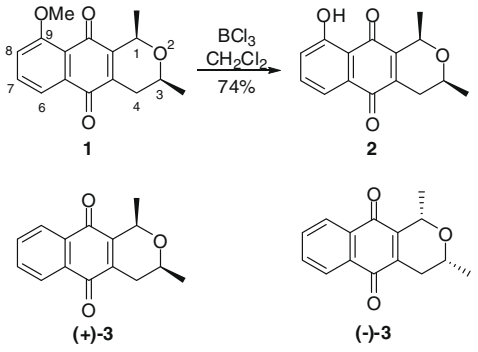
organisms and cancer cells.[17] The quinone group is also known to confer anti-TopoII activity in vitro.[18,19] Remarkably, it has been stated that quinones form a new mechanistic group of compounds that combine the features of both poisons and pure catalytic inhibitors.[20–22] One specific class of quinones, known as the pyranonaphthoquinones are of interest to our research group[17,23] and are known to include members with anti-topoisomerase II activity, such as the lapachones and eleutherin.[21,24] Herein we report the broad level of topoisomerase II activity and cytotoxicity exhibited by eleutherin, together with a series of structurally similar natural products, and analogues thereof.

2. Chemistry

Recently, we published a concise synthesis of the reversible topoisomerase II inhibitor (+)-eleutherin[21,25–27] **1** using a Hauser-Kraus annulation[28–31] as the key step. Our own long term interest in the synthesis of pyranonaphthoquinones[17,32,33] has led to the successful synthesis of several structurally related analogues of (+)-eleutherin **1** that were envisaged to possibly possess anti-topoisomerase II activity.

Our initial focus was to evaluate the effect of the aromatic methoxy group present in eleutherin **1** for its TopoII activity. Thus, eluetherin **1** was treated with boron trichloride in dichloromethane delivering (+)-9-demethyleleutherin **2** in good yield. Furthermore, both enantiomeric deoxyanalogues of eleutherin (+)-**3** and (−)-**3**, both bearing no substituents on the aromatic ring, had been previously prepared in high enantiomeric excess in our laboratory using a microwave assisted enzymatic resolution of a racemic secondary alcohol as the key step[34] (Scheme 1).



Scheme 1.

Ventiloquinone L **4** is a pyranonaphthoquinone natural product isolated from the root bark of *Ventilago goughii*.[35] Surprisingly, no biological activity data is available for ventiloquinone L **4** and considering its structural similarity to eleutherin **1**, it was also subjected to screening as a potential topoisomerase II inhibitor. The synthesis of (+)-ventiloquinone L **4** had been previously achieved in analogous fashion to eleutherin **1** using a Hauser-Kraus annulation as the key step.[36] The same synthetic route also furnished 7-methoxyeleutherin **6**.[36]

Our recent synthesis[37] of the 3C-protease inhibitor (−)-thysanone[38] **8** was achieved using a toluate anion addition to a lactone.[39,40] During the total synthesis of **8**[37] several C-3 demethyl analogues of eleutherin **1** were prepared, namely **5**, **7**, **9** and **11**. The deoxythysanone analogue **10** has also been obtained using a similar chemoenzymatic route that was used for the synthesis of deoxyeleutherin analogues (+)-**3** and (−)-**3**[34] (Fig. 1).

Nanaomycin A[41,42] is another pyranonaphthoquinone natural product of significant interest in our laboratory due to its wide range of biological activity,[41,43,44] however to the best of our knowledge it has not been screened for topoisomerase II inhibition. Our recent synthetic efforts towards nanaomycin A resulted in the enantioselective synthesis of a 1,3-*cis* analogue of deoxynanaomycin A (+)-**13**[45] using an asymmetric allylation as the key step. The racemic C8-oxygenated nanaomycin A analogue (±)-**12** was also obtained via a similar route.[46] Testing **12** and **13** against topoisomerase II was envisioned to probe the effect of the carboxylic acid side chain present at C3, a common structural feature in several pyranonaphthoquinone natural products[17,32,33] (Fig. 2).

The cardinalins are a series of C8-dimeric pyranonaphthoquinones that exhibit significant cytotoxic activity against P388 murine leukaemia cell lines.[47,48] Despite this promising cytotoxicity, the cardinalins (or any other dimeric pyranonaphthoquinones) have never been screened for specific TopoII activity. The synthesis of the dimeric pyranonaphthoquinone core of the cardinalins **14** was recently accomplished in this laboratory using a palladium catalysed homocoupling of an aryl triflate as a key step.[49] A regioisomer of the cardinalins **15**, in which the biaryl bond is at C6 instead of C8, has also been synthesised during our initial attempts[36] to effect a biomimetic dimerisation of ventiloquinone L **4** (Fig. 3).

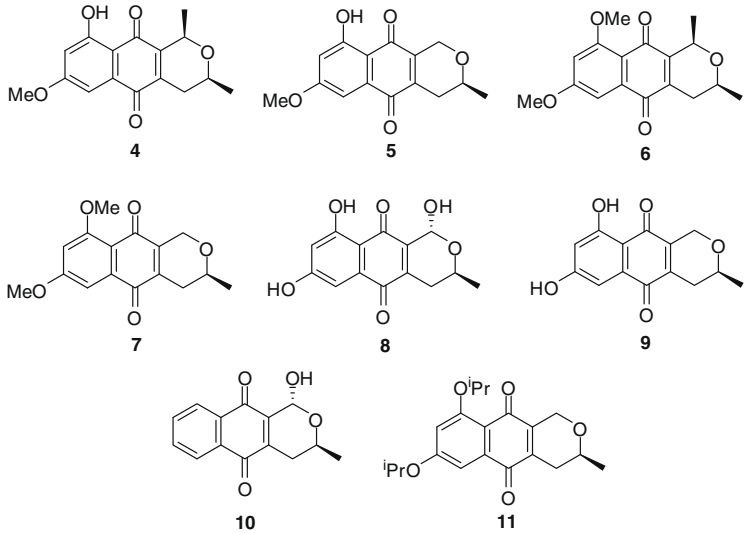


Figure 1.

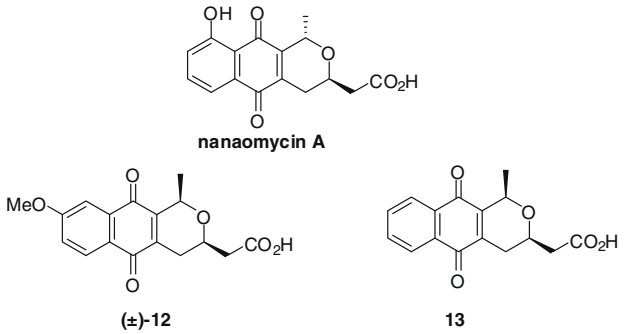


Figure 2.

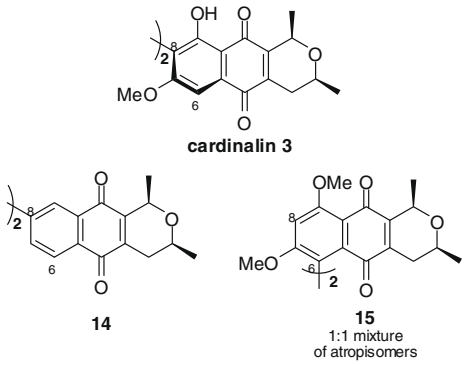


Figure 3.

3. Biological results and discussion

As eleutherin **1** and other pyranonaphthoquinones have been shown to inhibit/poison topoisomerase II,[21,23] we first evaluated the anti-topoisomerase II properties of our compounds **1–15** in vitro. We employed purified human topoisomerase IIα (hTopIIα), a key target of many antitumour drugs, and a set of small molecules of circular DNA as substrates in a series of different experiments. We measured the inhibitory potential of the above-mentioned compounds using two well-established strategies: decatenation of kinetoplast DNA and relaxation of a plasmid with a 'hot spot' for hTop2α.[50]

The decatenation assay is the most specific test for topoisomerase II activity. It employs a network of thousands of interlocked closed circular DNA molecules called kinetoplast DNA (kDNA) obtained from protozoa *Crithidia fasciculata*. Because of its overall size, kDNA cannot enter a typical agarose gel during electrophoresis, thus it remains in the loading well. However, topoisomerase II can release the individual small circular DNA molecules from the network while preserving its circular nature. Once released, these so-called 'minicircles' can enter the agarose gel and appear as a mixture of covalently closed topoisomers and nicked products. To simplify the overall number of visible products, the electrophoresis is carried out in the presence of an excess of an intercalating agent (ethidium bromide in these assays) to convert all covalently closed topoisomers to a unique band of maximum supercoiled unique topoisomer.

Thus, we incubated the purified hTop2α enzyme with the synthesised pyranonaphthoquinones and immediately added the kDNA (Fig. 4A). Two controls were included to confirm the correct performance of the assay. First, samples with just the final concentration of the solvent (1% DMSO) were used with or without the enzyme. These samples were also used to titrate the amount of enzyme to just complete the reaction for the indicated incubation time (i.e., 0.2 units of enzyme and one hour, respectively, not shown). Thus any minor inhibitory kinetics of the tested compounds could be detected and quantified. Decatenation is measured as the ratio between the minicircles two major bands and the intact kDNA. In Figure 4A we show the quantitation of this assay for all the pyranonaphthoquinones studied. We can see that all were capable of inhibiting hTop2α. Most of the compounds inhibit decatenation of kDNA strongly (virtually no topoisomerase activity), whereas some compounds did so in a weaker manner. Surprisingly, we noted that amongst these weak inhibitors were the actual natural products, ventiloquinone L **4** and thysanone **8**. This was interesting for two reasons. It may mean that natural pyranonaphthoquinones are naturally occurring privileged structures pre-selected by Nature to not interact with topoisomerases. Chemically, it seems that minor changes to the structure of the compounds could switch their anti-topoisomerase properties. Given the importance of determining the factors that makes this class of compounds good or bad topoisomerase II inhibitors we decided to further explore their anti-topoisomerase activity using a different approach.

We performed a more complex topoisomerase II assay based on the relaxation of a plasmid (pRYG) that contained a hot spot sequence for hTopIIα.[50] As they are present in bacteria, plasmids are negatively supercoiled in their natural form, mainly forming monomers ('SC' band in the agarose gel pictures, Fig. 4). Moreover, during the DNA extraction protocols, two more species are marginally obtained as well: a 'relaxed' covalently closed form ('OC' band) and a 'nicked' single-strand break form (termed 'Nckd'). All these topological species can be resolved in an agarose gel electrophoresis under specific conditions. Topoisomerase II can relax supercoiling in vitro to a set of topoisomers. All these complex reactions occur at different rates. In an 'end-point' reaction, conditions are set up to only reach the final products, the fully relaxed and nicked monomers. The amount of enzyme needed to reach the 'end-point' was titrated for the controls without chemicals but in the case of eleutherin **1** to a point where partial activity was observed. By adopting this approach, it could be evaluated whether any of our synthetic derivatives were better than this previously described anti-topoisomerase II compound, eleutherin **1**. As depicted in Figure 4B (upper panel), eleutherin **1** did not inhibit hTop2α as strongly as the well-known anti-topoisomerase II agents ellipticine and etoposide under our conditions. As expected from the decatenation assay, the other natural products (**4** and **8**) were even weaker inhibitors. Interestingly however, some of the derivatives were capable of inhibiting hTop2α almost completely (**5**, **7**, **9**, **11**, **12**, **14**, **15**). Thus, they seem to be more effective inhibitors of hTop2α than the natural product eleutherin **1** itself.

Thus, several conclusions can be reached from the two anti-TopoII assays used. The removal of the C9-methyl group from eleutherin **1** delivering the phenol **2** appears to have a negligible effect on activity. Interestingly, demethoxyeleutherin (+)-**3** is less active than the parent compounds **1** and **2** strongly suggesting the oxygenated substituent at C9 is essential for activity. Also, analogue (−)-**3** possesses slightly stronger inhibitory activity than its enantiomer (+)-**3** suggesting the stereochemistry of the *cis*-1,3-methyl group present on the pyran ring may also play a critical role in the mechanism of inhibition. Synthesis of the enantiomers of **1** and **2** would further confirm this postulate. Remarkably, the potent inhibitors **5**, **7**, **9** and **11** are all unsubstituted at C1 suggesting the presence of a methyl group in this position hinders activity. Furthermore, substituting the C1 methyl for a lactol (**8** and **10**) appears to reduce the activity rather drastically and the difference in activity between **12** and **13** suggests the methoxy group present at C8 improves activity in this case, although the existence of **12** as a racemate may also be a contributing factor.

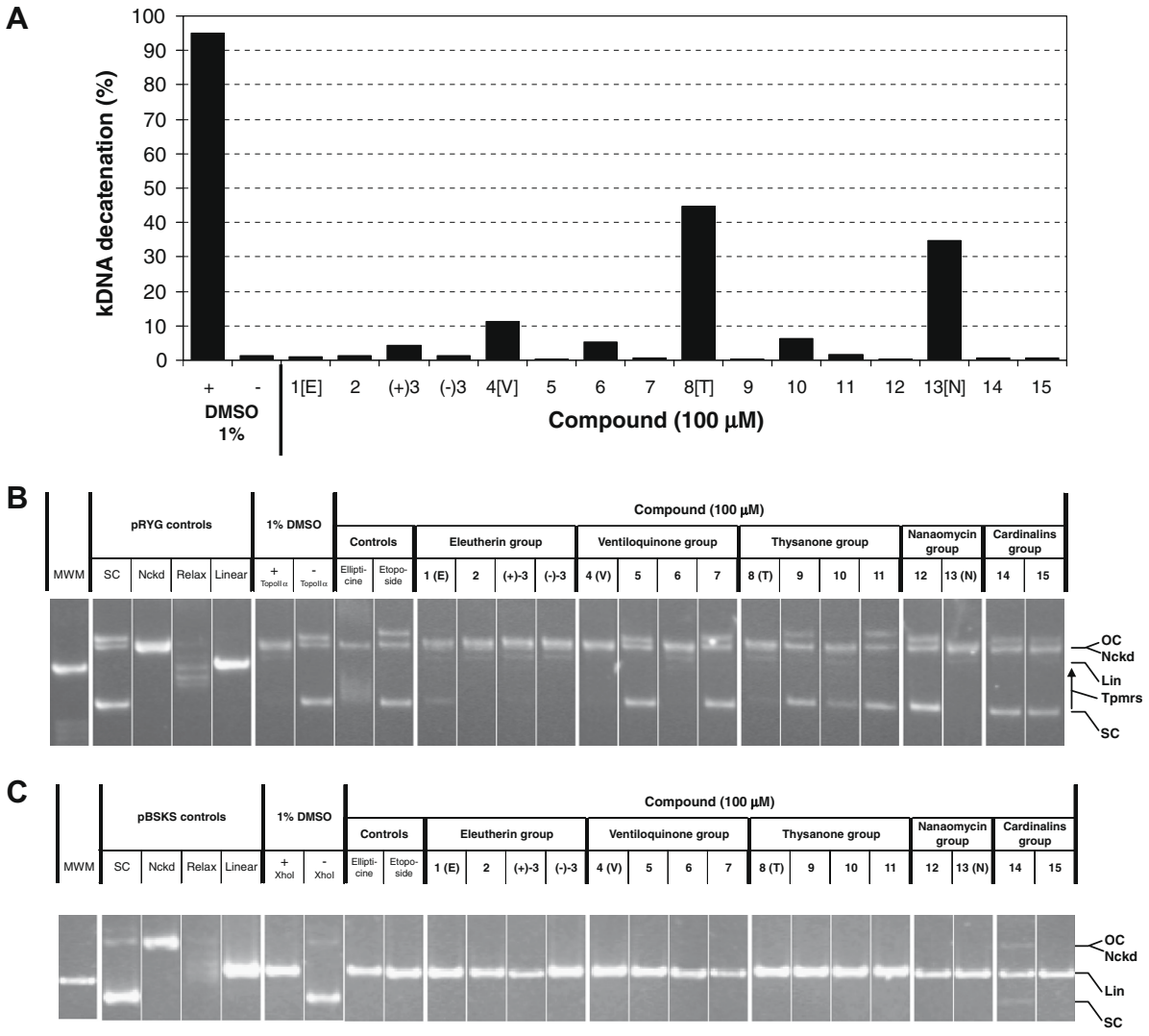


Figure 4. Specific inhibition of hTopoIIα by eleutherin **1**, ventiloquinone L **4**, thysanone **8**, deoxynanaomycin A **13** and their pyranonaphthoquinone derivatives. (A) Quantitation of a decatenation assay performed with low enzyme amounts. Catenanes minicircles from kinetoplast DNA were treated with 0.3 units of hTopoIIα in the presence of either just 1% DMSO ('+' bar), or 100 μM of the pyronaphthoquinones in 1% DMSO. A control with just the solvent and without the enzyme is also included ('−' bar). Note how the presence of all reported pyranonaphthoquinones at least partly inhibit hTopoIIα (as compared to control with just 1% DMSO). (B) Agarose gel of a relaxation assay with monomeric pRYG plasmid. Covalently closed negative supercoiled pRYG plasmid (SC form) was treated with hTopoIIα in the presence of either just 1% DMSO, or 100 μM of ellipticine, etoposide or the tested pyranonaphthoquinones in 1% DMSO. Note how the presence of pyranaphthoquinones **5**, **7**, **9**, **11**, **12**, **14** and **15** greatly inhibit hTopoIIα (as compared to controls with eleutherin **1**, ventiloquinone L **4**, thysanone **8** and deoxynanaomycin A **13**); however none intercalate within the DNA (SC forms migrate more slowly only in the ellipticin lane). (C) Pyranonaphthoquinones **1**–**15** do not unspecifically inactivate enzymatic activity. Covalently closed negative supercoiled pBSKS plasmid was treated with the endonuclease XhoI under the hTopoIIα buffers with or without the reported drugs. Nckd (nicked plasmid), OC (fully relaxed plasmid), Lin (linearised plasmid), SC (supercoiled plasmid), Tprms (relaxed monomeric topoisomers).

Significantly, the strong inhibition displayed by **14** and **15** establishes for the first time that dimeric pyranonaphthoquinones are in fact TopoII inhibitors. Overall, there appears to be a correlation between the presence of a substituent at C7 and C1; the presence of an oxygenated substituent at C7 diminishes the inhibitory activity when present in conjunction with a C1-methyl group but oxygenation on the aryl ring appears to increase inhibitory activity when the C1-methyl group is absent.

Next, we further tested whether any of the pyranonaphthoquinones were intercalating agents. For this purpose the intercalating agent and strong TopoII inhibitor ellipticine was included in the relaxation assays (Fig. 4B). We conclude that none of the studied pyranonaphthoquinones intercalate within the DNA (note how the SC forms migrate more slowly only in the presence of ellipticine).

Finally, we ruled out whether any of these pyranonaphthoquinones act as unspecific inactivators of proteins in vitro. We considered it important to address this issue because a mechanism of inactivation by quinones has been reported involving their reactivity with thiol groups.[20] In order to test this, we employed an endonuclease assay in which a restriction enzyme cuts the plasmid pBSKS to give a linearised form of it. This enzyme, XhoI, is active in the same hTop2α buffer in which all of the above experiments were performed. We could establish that the endonuclease was fully active in the presence of all of the pyranonaphthoquinones studied, hence reactivity with thiol groups was not considered a likely mechanism of action for TopoII interaction by the pyranonaphthoquinones under evaluation (Fig. 4C).

3.1. Growth inhibition features of pyranonaphthoquinones against *Saccharomyces cerevisiae*

Having shown clear differences in the anti-TopoIIα activities between the different pyranonaphthoquinones analysed, we in-

tended to correlate this with the putative in vivo cytotoxicity. We decided as a first approach to use the yeast *Saccharomyces cerevisiae* as a cell model system because it offers substantial advantages to elucidate the molecular mechanism of action of drugs within the cell as well as providing information on some important putative pharmacological features such as drug metabolism and permeability.[51,52]

We therefore studied the inhibition of yeast growth by the different compounds. A fresh dilute culture of a reference wild type strain of *S. cerevisiae* was monitored for growth after 24 h in the presence of different concentrations of the pyranonaphthoquinones. As it can be seen in Figure 5A, the natural pyranonaphthoquinones exhibited a diverse pattern of inhibition (although deoxynanaomycin A **13** is not a natural product, it is included in Fig. 5A for comparative purposes due to its carboxylic acid side chain). Thus, eleutherin **1** did inhibit growth by half (MIC_{50}) at a concentration of ~80 μM, whereas ventiloquinone L **4** and deoxynanaomycin A **13** were strongly cytotoxic with a MIC_{50} of around 5–10 μM. By contrast, thysanone **8** was not cytotoxic at all. When compared to all the compounds tested herein (Fig. 5B), it could be concluded that all eleutherin derivatives (**2**, (+)-**3** and (−)-**3**) were more cytotoxic than eleutherin **1** in spite of being slightly worse hTop2α inhibitors. Ventiloquinone L **4** and its derivatives (**5**–**7**) gave the most striking result as they were either effective cytotoxic compounds (**4** and **5**) with opposite hTop2α inhibition patterns or the other way around (**6** and **7**). On the other hand, closely related thysanone **8** derivatives (**9** and **10**) were also more cytotoxic and they were also better hTop2α inhibitors than **8**. A possible explanation between the difference in activity between **8** and **10** is as follows. Interestingly, compound **8** possesses two phenolic groups and compound **10** has none. Both compounds, **8** and **10**, have a lactol group but in the case of compound **10** a hydrogen bond between the hydroxyl group and the carbonyl group of the quinone is possible, thus helps stabilising the lactol ring. Meanwhile, for compound **8** there are two possibilities to form the hydrogen bond. In this case the ring-chain tautomerism of the lactol is feasible and the formation of a formyl group also is possible (although the ring opened product was not visible by NMR).

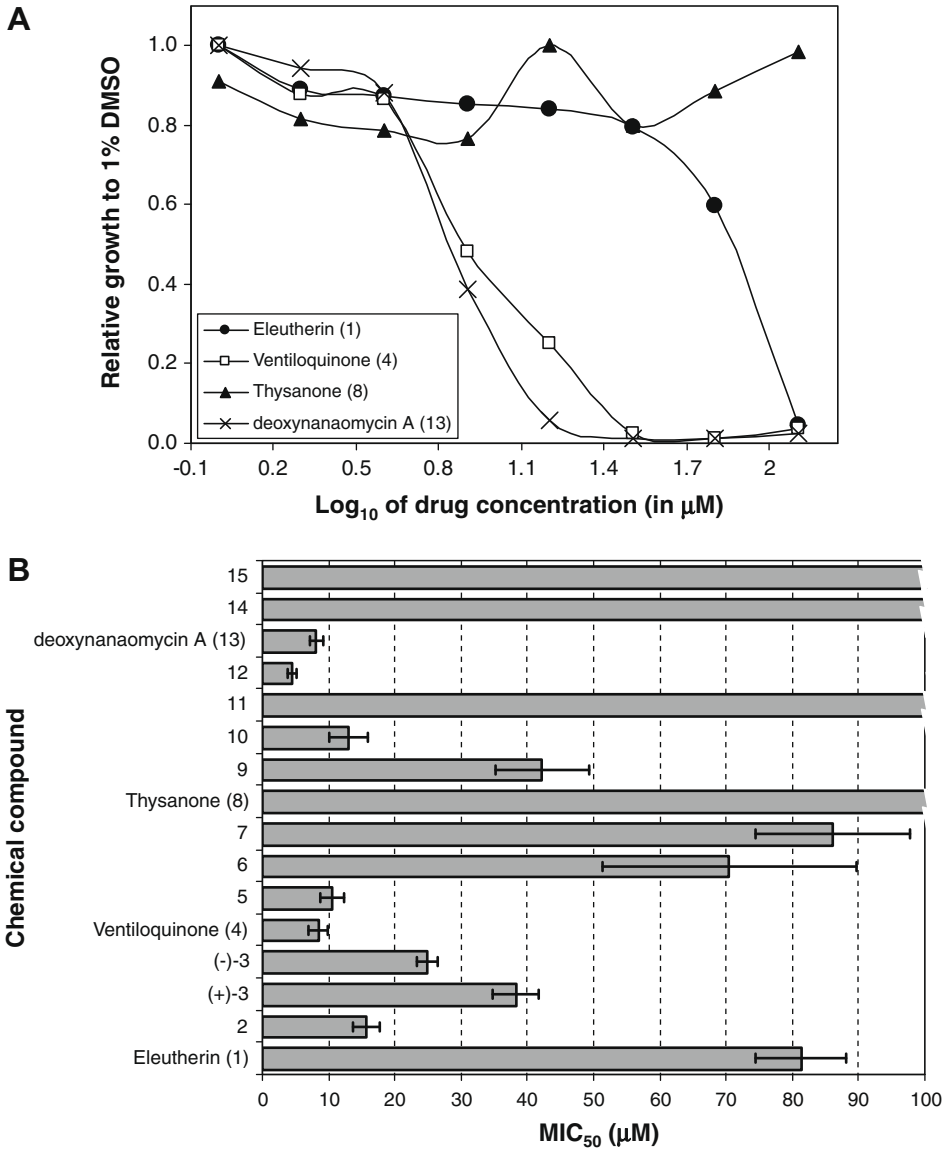


Figure 5. In vivo growth inhibition of *Saccharomyces cerevisiae* by eleutherin **1**, ventiloquinone L **4**, thysanone **8**, deoxynanaomycin A **13** and their pyranonaphthoquinone derivatives. (A) Relative growth curves to different concentrations of eleutherin **1**, ventiloquinone L **4**, thysanone **8** and deoxynanaomycin A **13**. (B) Concentrations that inhibit yeast growth by 50% for all the compounds studied. Mean and SEM of four independent experiments.

As for deoxynanaomycin A **13** and its analogue **12**, they were the most cytotoxic compounds of all, indicating the carboxylic acid side chain at C3 plays a pivotal role in this effect, although they also differed significantly in the hTop2α inhibition. The difference in TopoII activity between **12** and **13** further suggests that oxygenation on the aryl ring promotes TopoII activity. Finally, the dimeric cardinalin derivatives **14** and **15** were found to be very strong hTop2α inhibitors while essentially being non-cytotoxic to yeast. However, in this case it is probable that their large molecular mass prevents them entering the cell.

4. Conclusions

In conclusion, we have probed the topoisomerase II inhibitory properties of synthetic and natural analogues of the known topoisomerase II inhibitor (+)-eleutherin **1**, discovering several derivatives more potent than the natural product **1**. It appears from our results that the nature of the substituent at the C1 position and the oxygenation on the aryl ring are essential for increased activity. We have also reported for the first time the ability of nanaomycin derivatives and dimeric pyranonaphthoquinones as effective anti-TopoII agents, thus furthering the potential of this class of compounds.

From the comparison between yeast cytotoxicity and topoisomerase II inhibitory properties a diverse series of results were obtained. For all of the compounds investigated it was shown that some were weak TopoII inhibitors but strongly cytotoxic (**4**, **10**, **13**), suggesting a separate mechanism of cytotoxicity is operating other than TopoII inhibition. Furthermore, some of the compounds were potent TopoII inhibitors but possessed little to no cytotoxicity against yeast (**11**, **14** and **15**). Thus, we can conclude that other chemical features need to be considered in order for the pyranonaphthoquinones evaluated to be considered useful and specific anti-TopoII drugs in vivo. Factors such as intracellular metabolism of the compounds by the set of quinone oxido-reductases,[53,54] permeability to cell membranes,[51] species selectivity for the target, and other cytotoxic mechanisms such as ROS production[55] should also be taken into account.

Further work towards the synthesis of pyranonaphthoquinone derivatives based on the potent topoisomerase II inhibitors discovered herein, as well as studies to further understand the interesting link between TopoII inhibition and cytotoxicity by this class of compounds is in progress and will be reported in due course.

5. Experimental

Full experimental detail of all tested compounds **1–15** can be found in the Supplementary data.

5.1. Biology

5.1.1. Biological assays

The solvent for all the stocks of the chemical agents employed was dimethylsulfoxide (DMSO), special Molecular Biology grade (DNase and RNase-free), from Sigma–Aldrich. Ellipticine and etoposide were purchased from Sigma–Aldrich and stored as 10 mM in DMSO stock at −20 °C. Synthetic pyranonaphthoquinones were also stored in DMSO as a 10 mM stock at −20 °C until their use.

5.1.2. TopoII-mediated DNA decatenation assay

The TopoII assay kits from TopoGEN (Columbus, OH) were used. Also purified hTopoIIα was purchased from the same vendor. DNA decatenation assays were performed according to the manufacturer's instructions and earlier procedures with some minor modifications. The assay was performed in a total reaction volume of 20 μl containing 50 mM Tris–HCl (pH 8.0), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM ATP, 30 μg/ml bovine serum albumin and 150 ng of kDNA. All chemical compounds were then added to a final concentration of 100 μM in 1% DMSO. Reactions were initiated by addition of 0.3 units of hTopoIIα, and incubated for 60 min at 37 °C. The reaction was terminated by the addition of 5 μl of stock solution (5% SDS, 25% Ficoll and 0.05% bromophenol blue) followed by treatment with 0.25 mg/ml proteinase K (Roche) at 55 °C for 30 min to eliminate the protein. Samples were resolved by electrophoresis on a 1% (w/v) agarose gel containing 0.5 μg/ml ethidium bromide in TAE buffer (100 mM Tris–acetate and 2 mM Na_2EDTA, pH 8.3) also containing ethidium bromide at the same concentration. DNA bands were visualised by UV and photographed and documented with QuantityOne™ (BioRad).

For quantitation of the substrate (kDNA) and products of the TopoII reaction (Nck, SC & catenanes), the numerical values of intensity profiles of the different lanes were obtained after background substraction and exported to Microsoft Excel™. For each lane, the overall intensity was used to obtain the fraction of intensity for the different bands (kDNA, Nckd & SC). Here we measured hTopoIIα activity based on the ratio of final products (nicked plus supercoiled minicircles) versus substrate (kDNA).

5.1.3. TopoII-mediated DNA relaxation assay

The TopoII drug kit from TopoGEN (Columbus, OH) was used. The 20 μl reaction mixture contained 250 ng of pRYG plasmid DNA and 0.5 mM ATP in the assay buffer [10 mM Tris–HCl, 50 mM KCl, 50 mM NaCl, 0.1 mM EDTA, 5 mM $MgCl_2$ and 2.5% (v/v) glycerol, pH 8.0]. Plasmid was extracted using a Midiprep kit (Qiagen) from *Escherichia coli* JM109 strain grown to saturation. Chemical compounds were at 100 μM in 1% DMSO. The reaction components were added as follows: assay buffer, DNA, chemical compound from DMSO stock or just DMSO, and finally 3 units of hTopoIIα. The reaction mixture was incubated at 37 °C for 60 min, and quenched with 1% (w/v) SDS and 25 mM Na_2EDTA. The mixture was treated with 0.25 mg/ml proteinase K (Roche) at 55 °C for 30 min to digest the protein. pRYG DNA species were resolved in 1% (w/v) agarose gel electrophoresis in 1× TBE buffer (89 mM Tris–borate and 2 mM Na_2EDTA, pH 8) without ethidium bromide. The photograph was taken after staining with ethidium bromide.

5.1.4. Endonuclease inactivation assay

Plasmid pBluescript was used instead of pRYG. Protocol was performed as for the TopoII-mediated relaxation assays except for the use of endonuclease XhoI (1 unit) instead of hTopoIIα.

5.1.5. Yeast in vivo growth experiments

Reference *S. cerevisiae* haploid strain BY4741 from Euroscarf was used for all experiments. This strain was grown overnight at 30 °C in an orbital incubator from fresh preinocules in YPD media (yeast extract 1%, peptone 2% and dextrose 2%). While in the logarithmic phase of growth (~0.6 optical density units at 660 nm per ml); the culture was split and transferred to fresh YPD media at a final concentration of 0.01 OD_{660}/ml. Chemical compounds were added from stocks to give the following final concentrations (in 1% DMSO): 1, 2, 4, 8, 16, 32, 64 and 128 μM, respectively. A control of YPD media plus DMSO (1% v/v) was also used. All cultures were put to grow at 30 °C for 24 h and then OD_{660}/ml was taken as a measure of growth. After normalisation to the DMSO (1% v/v) control, relative growth was plotted against the $\log_{10}$ of the chemical concentration and the MIC_{50} (minimum concentration for a 50% growth inhibition) was calculated according to the strategy developed by Casey et al.[56]

Acknowledgements

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Supplementary data

Supplementary data (full experimental details and HPLC data of all tested compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.08.064.

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