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Salen–Anthraquinone Conjugates. Synthesis, DNA-Binding and Cleaving Properties, Effects on Topoisomerases and Cytotoxicity

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Abstract—A series of amidoethylamino-anthraquinone derivatives bearing either one or two salen (bis(salicylidene)ethylenediamine) moieties complexed with Cu^{II} or Ni^{II} have been synthesized, and their DNA-binding and cleaving properties examined. The effects of the mono- and di-substituted anthracenedione–salen conjugates on DNA cleavage mediated by topoisomerases I and II have also been determined, as well as their cytotoxicity toward human KB cells. The anthraquinone–salen•Ni^{II} conjugates bind to GC-rich sequences in DNA, but do not cleave the macromolecule. By contrast, the anthraquinone–salen•Cu^{II} hybrids do not recognize particular nucleotide sequences but efficiently induce single-strand breaks in DNA after activation. The 5,8-dihydroxy-anthraquinone conjugates are more cytotoxic and more potent toward topoisomerase II than the non-hydroxylated analogues, but they are less cytotoxic than the salen-free anthraquinones. The attachment of a salen•Cu^{II} complex to the anthraquinone chromophore can confer DNA cleaving properties in vitro, but this is at the expense of cytotoxic activity. Anthraquinone–salen•Cu^{II} complexes may find useful employ as footprinting probes for investigating ligand–DNA interactions. Copyright © 1996 Elsevier Science Ltd

Introduction

Anthracenediones represent an important class of antitumour agents.[1–3] Mitoxantrone (Fig. 1), which is the lead compound in the series, is routinely used in the clinic for the treatment of certain hematological malignancies, as well as for ovarian and breast cancers.[4–8] In common with the anthracyclines, the mechanism by which antitumour anthracenediones exert their cytotoxic activities is multimodal and involves (1) interaction with DNA[9–13] (preferentially at pyrimidine-3′,5′-purine GC-rich sites)[14–16] and nucleic acid condensation,[17] (2) inhibition of topoisomerase II activity leading to DNA cleavage,[18,19] and (3) production of DNA damaging free radicals.[20–23] As with many DNA intercalating drugs (such as anthracyclines or amsacrine), topoisomerase II-dependent DNA cleavage is thought to be the critical event responsible for the anticancer activity; whereas, free radical-mediated DNA cleavage is probably more closely related to the cardiotoxic side effects of mitoxantrone.[24] In the past 10 years numerous anthracenedione derivatives have been synthesized with the aim of finding related compounds showing better therapeutic efficacy together with fewer side effects.[25–27] The chemical modifications of mitoxantrone, which have commonly been considered, concern either the nature and position of the aminoacyl side chains[28–35] or the chromophore unit (e.g., azaanthracenediones, anthrapyrazoles).[36–39] An alternative strategy involves attaching the anthraquinone chromophore to a metal complex capable of triggering DNA cleavage. Anthraquinone derivatives linked to the copper-chelating peptides GGH and GHK have been synthesized previously.[40–42] A conjugate bearing two GGH•Cu^{II} moieties at positions 1 and 4 of the anthracenedione chromophore has shown potent cytotoxic activity in vitro and was found to be more active than mitoxantrone against P388 leukemia in mice.[42] The antitumour activity of the anthraquinone–bis(GGH•Cu^{II}) hybrid molecule was attributed to its propensity to bind tightly to DNA and to induce DNA cleavage via the production of oxygen-based free radicals. These promising results, together with findings reported by us and others on acridine–metal complexes[43–46] and bleomycin models,[47–49] suggest that the concept of attaching a metal complex to an intercalating chromophore is valid and warrants further development.

In the present article, we report the synthesis of novel aminoalkylamino 1,4-anthracene-9,10-diones substituted with one or two tetradentate copper- and nickel-chelating ligands structurally related to salen [bis(salicylidene)ethylenediamine].[50] Complexes of salen with Ni^{II} or Cu^{II} (but also Mn^{II}) can induce DNA cleavage under reducing conditions.[51–54] The linkage of salen•metal complexes to the side chains of mitoxantrone are expected to confer DNA cleaving properties in much the same way as previously shown with GGH–anthraquinone conjugates. Here we describe the DNA-binding and cleaving properties, the effects on topoisomerase II in vitro, and the production of oxygen radical species by the Cu^{II} or Ni^{II} complexes of the mono- and bis-substituted anthraquinone–salen conju-

gates (Fig. 1). Preliminary biological studies comparing the cytotoxic activities of the conjugates with those of the parent compounds towards a human KB cell line are also provided.

Results

Chemistry

The synthesis of the salen•Cu^{II} complex **6** has recently been reported.[55] A similar strategy was used to synthesize the salen•Ni^{II} complex **8** (Scheme 1). Briefly, treatment of the commercially available *N*α-Z-*N*ε-Boc-L-lysine (**1**) with ethyl chloroformate in ammonia-saturated THF gave the carboxamide **2**, which was then dehydrated with trifluoroacetic anhydride in the presence of triethylamine to provide the nitrile **3**. Hydrogenation of **3** over Raney nickel gave the asymmetric α,β diamine **4**.[56] The benzyloxycarbonyl (Z) protecting group was cleaved during this reaction (hydrogenolysis). The α,β diamine **4** was then condensed with salicylaldehyde in the presence of cuprous acetate monohydrate or nickel acetate tetrahydrate to afford the Boc'-protected copper and nickel complexes **5** and **7**, respectively. The Boc'-protecting group of compounds **5** and **7** was removed under acidic conditions to yield the corresponding amines **6** and **8**.

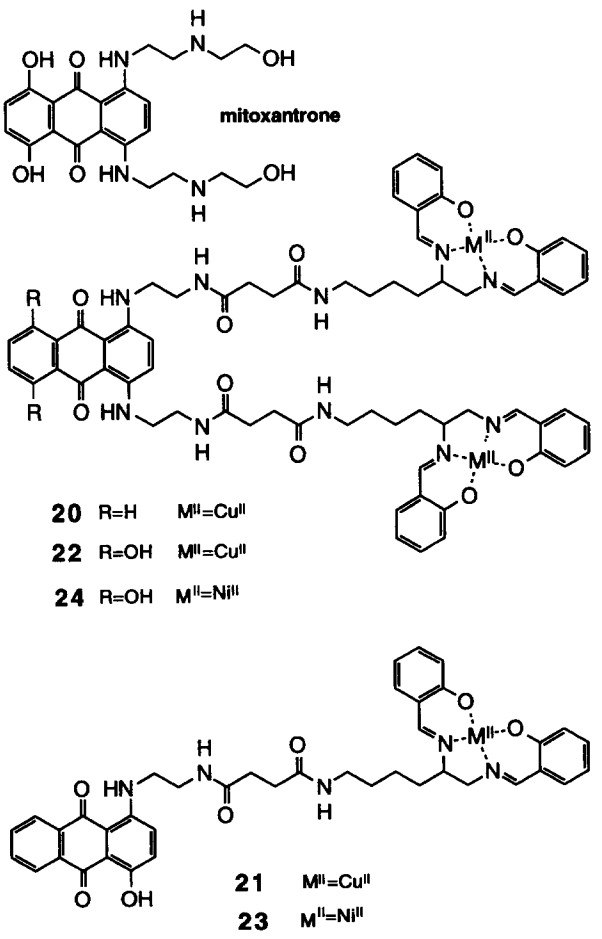


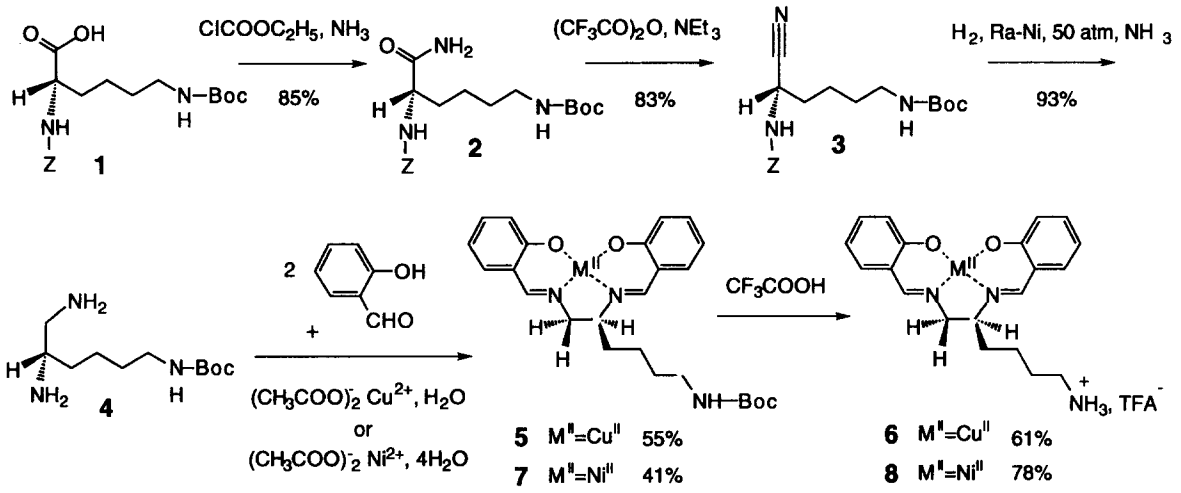
Figure 1. Chemical structure of mitoxantrone and the salen–anthraquinone conjugates.

The chemical procedure outlined in Scheme 1 has general applicability for other amino acids.

The synthesis of the anthraquinone–salen conjugates is depicted in Scheme 2. The anthracenedione **11** and dihydroxyanthracenedione **13** were prepared by condensation of leucoquinizarine **9** or 5,8-dihydroxy-leucoquinizarine **10** with Boc'-ethylenediamine followed by air oxidation of the dihydro intermediates, as previously described.[42] The mono-substituted anthraquinone derivatives are also formed during the reaction, but only the non-hydroxylated mono-substituted compound **12** could be isolated and purified by column chromatography. Cleavage of the Boc-protecting group from the mono- and bis-substituted aminoethylamino-anthraquinones **11–13** afforded compounds **14–16**, which were then treated with succinic anhydride in the presence of pyridine to produce the corresponding succinyl-aminoethylamino-anthraquinone derivatives **17–19**. Finally, the anthraquinone–salen•Cu^{II} complexes **20**, **21**, and **22** were obtained by condensation of the anthraquinones **17**, **18**, or **19** with the salen•Cu^{II} complex **6** via a conventional coupling procedure using dicyclohexylcarbodiimide (DCC) and *N*-hydroxybenzotriazole (HOBt). The same method was applied using the salen•Ni^{II} complex **8** to obtain the anthraquinone–salen•Ni^{II} conjugates **23** and **24**. Various methods were tried to synthesize the non-hydroxylated anthraquinone substituted with two salen•Ni^{II} complexes, but this compound could not be obtained.

Binding to DNA

Two DNA fragments of 117 and 265 base pairs were cut from plasmid pBS with the restriction enzymes *Eco*RI and *Pvu*II; each was prepared with one of the complementary strands labelled at the 3′-end with ^{32}P. DNase I was allowed to cleave the DNA fragment alone or after prior equilibration with the drugs under test. A typical autoradiogram from such an experiment, applied to the water-soluble anthraquinone derivatives **14** and **16**, is presented in Figure 2. With both compounds, the relative pattern of cleavage differs substantially from that seen in the drug-free (control) lanes, and there are several regions where the susceptibility to DNAase I cutting is significantly reduced (footprints). This convincingly demonstrates that both anthracenediones bind to DNA in a sequence-selective fashion. Band intensities from the different gel lanes were measured by densitometry and converted into differential cleavage plots (not shown). In agreement with the results reported for mitoxantrone,[14] we found that the cleavage at GC-rich sequences is often reduced, such as at the sequence GCATGCCTGC between nucleotide positions 39 and 48; whereas, regions of enhanced cleavage (sometimes quite pronounced) occur mainly at AT-rich sequences such as the octanucleotide AATTAAC between positions 73 and 79. The two anthraquinones differ little as regards their selectivity, but it is frequently the case that footprints are slightly more pronounced with the dihydroxy anthraquinone **16** than with analogue **14**,



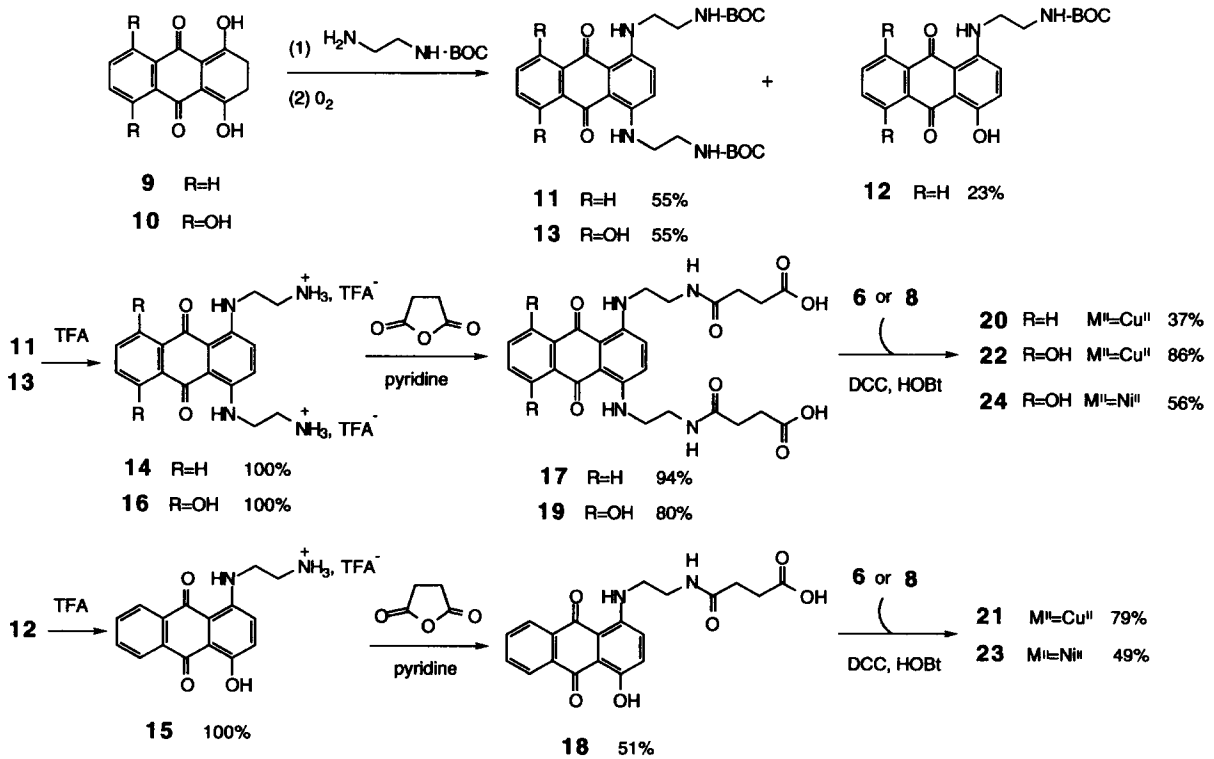
Scheme 1.

which lacks the OH groups on the anthracenedione chromophore. The footprinting data are consistent with preferred binding of the two anthraquinones to sequences containing the trinucleotides 5′-(A/T)CG or 5′-(A/T)CA, which are the consensus sequences recently determined for mitoxantrone.[16]

The salen•Ni^{II} complex **8** does not recognize any particular nucleotide sequence in DNA, but linkage to the anthraquinone chromophore confers sequence selectivity. The anthraquinone–(bis-salen)•Ni^{II} conjugate **24** and, to a lesser extent, its mono-substituted counterpart **23** exhibit a preference for GC-rich regions, such as the sequence 5′-ACGACGG around position 40 (Fig. 3). By contrast, no inhibition of cleavage by DNase I could be detected with any of the salen•Cu^{II} complexes, whether the copper complex was attached to the anthraquinone chromophore or not. The chemical nature of the chelated metal apparently influences the recognition of particular DNA sequences by the anthraquinones.

DNA cleaving properties

Strand scission was analyzed by monitoring the conversion of supercoiled plasmid DNA (form I) to nicked



Scheme 2.

circular molecules (form II) and linear DNA (form III). The tests were performed under aerobic conditions in the presence of a reducing agent, 2-mercaptopropionic acid (MPA) for the copper complexes and $KHSO_5$ (oxone) for the nickel complexes. None of the drugs can cleave DNA in the absence of a reducing agent. Although it has been reported that salen•Ni^{II} complexes can induce DNA cleavage when activated with oxone,[53] we could not detect any cutting of plasmid DNA with the nickel complexes **8**, **23**, and **24**. This is not really surprising since in previous studies with salen•Ni^{II} complexes, cleavage was observed at single-stranded regions of DNA (bulges and hairpins), but not within the B-form double-stranded helix.[52,53,57] By contrast, the salen•Cu^{II} complexes cleave DNA very efficiently (Fig. 4). Cutting of the plasmid by the salen•Cu^{II} ligands is most efficient when the activation is performed with mercaptopropionic acid (MPA), which is also the reagent of choice for DNA cleavage by phenanthroline•Cu^{II} complexes.[58,59] Much weaker cutting was observed using dithiothreitol, and very little cleavage at all was seen using hydrogen peroxide (data not shown).

Incubation of the plasmid for 2 h at 37 °C with increasing concentrations of the salen•Cu^{II} compound **6** causes conversion of form I to the nicked form II, but even with a drug concentration as high as 200 μM a small fraction of the supercoiled plasmid remains intact (Fig. 4, panel A). The salen•Cu^{II} compounds equipped with an anthraquinone chromophore are much more active than **6**, which lacks the intercalating moiety.

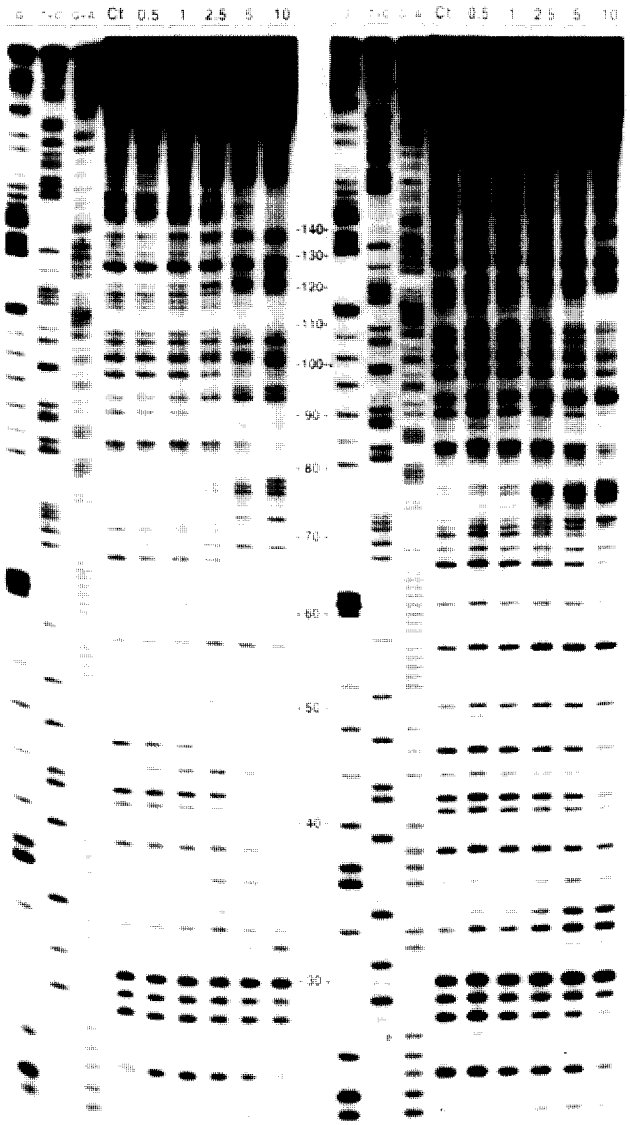


Figure 2. DNase I footprinting with the 265-mer *Pvu*II–*Eco*R I fragment of the plasmid pBS in the presence of various concentrations of the anthraquinone derivatives **16** (left panel) and **14** (right panel). The DNA was 3′-end labelled at the *Eco*RI site with α-^{32}P-dATP in the presence of AMV reverse transcriptase. Products of DNase of AMV reverse transcriptase. Products of DNase I digestion were resolved on an 8% polyacrylamide gel containing 8 M urea. The concentration (mM) of the drug tested is shown at the top of each gel lane. Control tracks labelled 'Ct' contained no drug. The tracks labelled T+C, G+A, and G represent Maxam–Gilbert sequencing markers specific for pyrimidine, purine, and guanine residues, respectively. Numbers between the two panels refer to the published nucleotide sequence of the fragment.[62]

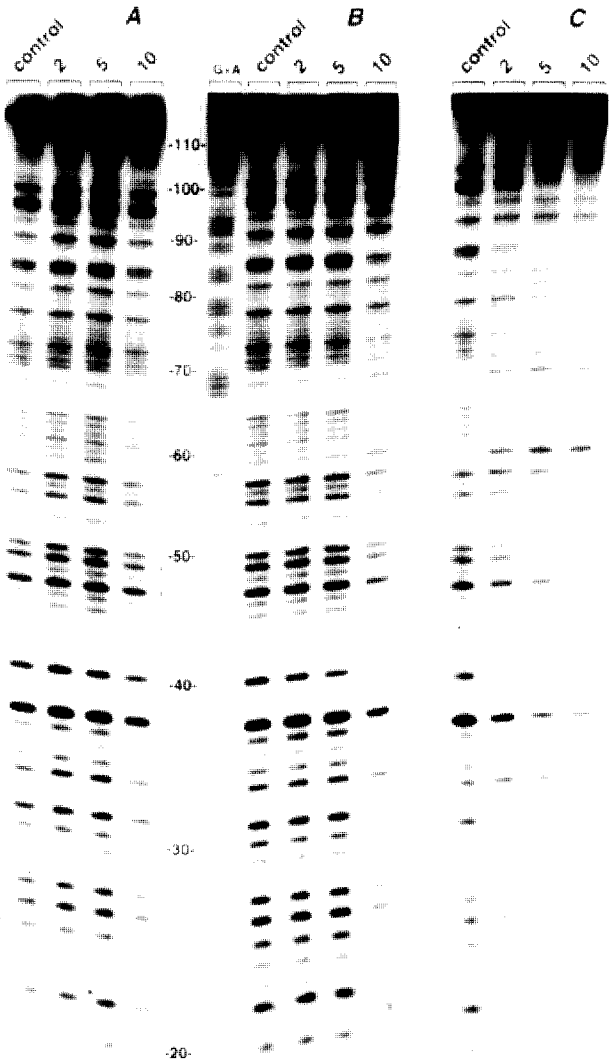


Figure 3. DNase I footprinting with the 3′-end labelled 117-mer fragment of the plasmid pBS in the presence of the salen•Ni^{II} complex **8** (a) and the anthraquinone–salen•Ni^{II} conjugates **23** (b) and **24** (c). Other details as for Figure 2.

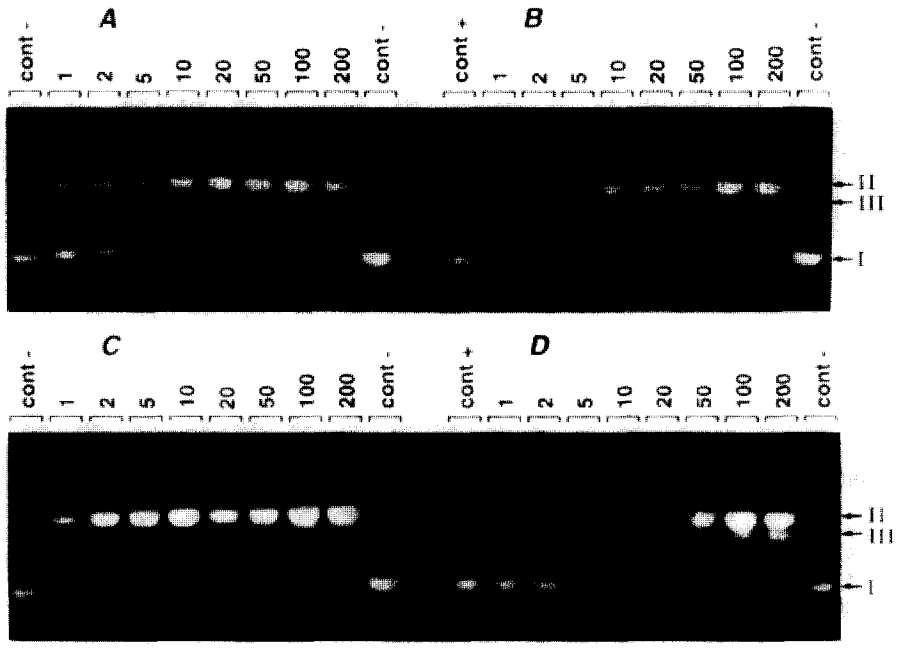


Figure 4. Cleavage of closed circular pUC12 DNA (form I) by the salen•Cu^{II} complex **6** (A) and the anthraquinone-salen•Cu^{II} conjugates **18** (B), **19** (C), and **17** (D) in the presence of 2-mercaptopropionic acid (MPA) as reducing agent. Forms II and III refer to the nicked and linear DNA forms, respectively. The drug concentration (μM) is indicated at the top of each gel lane. Control lanes marked cont + and cont − refer to the plasmid DNA incubated without drug in the presence and absence of MPA, respectively.

Indeed, with the three conjugates **20**, **21**, and **22** (Fig. 4, panels B, C, and D), a concentration of 50 μM is sufficient to cause the complete conversion of form I to form II. A small amount of linear DNA (form III) can be detected at high concentrations, particularly with the (bis-salen)–anthraquinone conjugates **20** and **22**. The appearance of linear DNA is attributed to the accumulation of single-stranded cleavages rather than to double-stranded cleavage. Introduction of a second salen•Cu^{II} complex on to the anthraquinone chromophore slightly reinforces the extent of DNA cleavage, but the presence or absence of the hydroxyl group on the anthraquinone ring apparently has little, if any, effect on the cutting efficiency. The anthraquinone–salen•Cu^{II} conjugates, thus, represent efficient tools for single-strand cleavage of DNA.

Oxygen radical species are most likely to be responsible for DNA cleavage by the salen•Cu^{II} complexes, as judged from ESR studies using 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) as a spin-trapping agent. DMPO was added to the salen•Cu^{II} compounds in an Me_2SO solution saturated with oxygen. Figure 5 [(b) and (c)] shows ESR spectra characteristic of DMPO–OH adducts (hyperfine splitting 1:2:2:1; $a_N = a_H = 14.87$ G). No such ESR signal was observed in the absence of the salen•Cu^{II} complex [Fig. 5(a)] nor with the salen•Ni^{II} complexes. The ESR signals obtained with the salen•Cu^{II} compound **6** and the anthraquinone–salen•Cu^{II} conjugates **17–19** are very similar, indicating that the different copper complexes produce roughly equal amounts of oxygen-based radicals. We did not observe any signal characteristic of the DMPO–CH_3 adduct (a signal consisting of six lines with equal intensities), which would be expected from the trapping of $CH_3^{\bullet}$ radicals formed by the reaction of Me_2SO with hydroxyl radicals $OH^{\bullet}$. We therefore conclude that the DMPO–OH adducts detected by ESR arise from the decomposition of DMPO–OOH adducts, which speaks for the production of superoxide anions $O_2^{-\bullet}$.

The postulated mechanism of DNA cleavage by the salen•Cu complex in the presence of MPA involves reduction of salen•Cu^{II} to salen•Cu^{I} which reacts with O_2 to give $O_2^{-\bullet}$ followed by the formation of H_2O_2. H_2O_2 would decompose upon reaction with DNA-bound salen•Cu^{I} to yield $OH^{\bullet}$ radicals capable of reacting with the deoxyribose residues of the DNA.

DNA-cleavage experiments were also performed with the ^{32}P labelled 117-mer and 265-mer DNA fragments

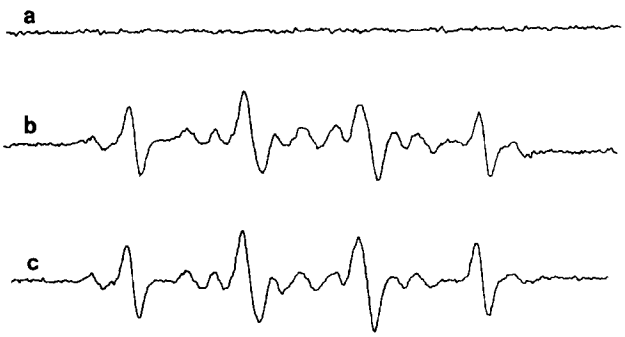


Figure 5. ESR spectra of DMPO adducts obtained from oxygenated aqueous solutions of DMPO in the absence (a) and presence of the salen•Cu^{II} complex **6** (b) and the anthraquinone–salen•Cu^{II} conjugate **22** (c). The drug solution (and the control) was bubbled with O_2 for 4 min prior to adding the DMPO. Experimental conditions: time constant 0.25 s, gain 1.25×10^4, microwave frequency 9.44 GHz.

used for footprinting to determine whether the anthraquinone–salen•Cu^{II} conjugates induce random or sequence-dependent breaks in DNA. The drugs were incubated with the radioactively labelled DNA, followed by MPA to initiate the cleavage reaction. After 2 h of incubation, the DNA cleavage products were separated on a denaturing polyacrylamide gel. No cleavage sites could be detected with the $KHSO_5$-activated salen•Ni^{II} complexes; whereas, with the MPA activated salen•Cu^{II} complexes, weak cleavage was found to occur at random, independent of the DNA sequence (data not shown). This result is entirely consistent with non-specific binding of the anthraquinone–salen•Cu^{II} conjugates to DNA as determined by footprinting.

Effects on topoisomerases in vitro

The effects of the salen–anthraquinone conjugates and their parent compounds on topoisomerase I and II (both purified from calf thymus) were studied using the ^{32}P labelled 4330 bp *Eco*RI-*Hin*dIII fragment of pBR322 as a substrate. The DNA cleavage products were analyzed by agarose gel electrophoresis under alkaline conditions (for topoisomerase I) or neutral conditions (for topoisomerase II). Drugs were tested at different concentrations varying from 0.1 to 100 μM. The inhibitory potency of the compounds was assessed by inspection of the autoradiograms and by comparing the cleavage of DNA by the enzyme in the absence and presence of the drug. The minimum drug concentration required to detect a stimulation of DNA cleavage is indicated in Table 1. Practically none of the compounds inhibit topoisomerase I. A slight induction of DNA cleavage was observed with the dihydroxyanthraquinone **16** and the mono-salen–anthraquinone **21**, but the effect is rather modest compared with the effect obtained with the reference topoisomerase I inhibitor camptothecin.[60] The salen-free anthraquinone derivatives **14** and **16** stimulate topoisomerase II-mediated DNA cleavage at 1 and 0.1 μM, respectively, although the concentration required to detect stimulation of cleavage is much higher than the concentration of mitoxantrone required to produce the same extent of cleavage (0.01 μM). The salen•Cu^{II} and salen•Ni^{II} complexes **6** and **8**, lacking the anthraquinone moiety, exert absolutely no effect on topoisomerase II. The linkage of the salen•metal complex to the anthraquinone chromophore significantly decreases the capacity of the drug to interfere with topoisomerase II, since stimulation of DNA cleavage with the salen conjugates **20** and **21** lacking the hydroxy groups on the anthraquinone ring could be detected only at the highest drug concentration (100 μM). However, cleavage stimulation was observed at concentrations as low as 1 μM with the two (bis-hydroxy)anthraquinone–salen Cu^{II} and Ni^{II} complexes **22** and **24**.

Table 1. Effects on topoisomerases I and II in vitro, and cytotoxicity of the salens and anthraquinone–salen conjugates towards KB human carcinoma cells

	MIC (μM)[a]		
	Topo-I	Topo-II	IC_{50} (μM)[b]
6	>100	>100	>10
8	>100	>100	>10
14	>100	1	0.03
16	10	0.1	0.01
20	>100	100	>10
21	10	100	0.2
22	>100	1	0.3
23	>100	>100	1
24	>100	1	0.3
Mitoxantrone	>100	0.01	0.003
Camptothecin[c]	0.01	>100	0.016

[a]Minimum drug concentration required to stimulate DNA cleavage by topoisomerase.
[b]Drug concentration that inhibits cell growth by 50% after incubation for 96 h. Each drug concentration was tested in quadruplicate.
[c]Data from ref 61.

Cytotoxic activity

The cytotoxicity of the test drugs was examined using KB human epidermoid carcinoma cells exposed to each agent for 4 days. IC_{50} values are collated in Table 1. The anthracenedione derivatives **14** and **16** are about tenfold less cytotoxic than mitoxantrone and this is surely connected with the observation that they are tenfold less active on topoisomerase II in vitro than mitoxantrone. At first sight, it is tempting to speculate that the cytotoxic activities of the salen-containing compounds correlate with their capacity to stimulate DNA cleavage by topoisomerase II. Both the salen•metal complexes **6** and **8** and the salen–anthraquinone conjugate **20**, which fail to inhibit topoisomerase II, are not cytotoxic; whereas, the salen–anthraquinone conjugates **22** and **24**, which can stimulate DNA cleavage by topoisomerase II, kill the cells very efficiently. However, the anthraquinone **21** attached to only one salen•Cu^{II} complex is also cytotoxic, yet it has no effect on topoisomerases. For the anthraquinone–salen•Ni^{II} complex **23**, the cytotoxic activity may result from the additive effect against topoisomerases I and II.

Discussion

The results reported here show that linkage to the salen•metal complex diminishes the cytotoxicity of the anthraquinone derivatives. They therefore contrast with the results previously obtained with anthraquinone–peptide•Cu^{II} conjugates.[42] Attachment of the salen•Cu^{II} complex to the anthraquinone chromophore can confer DNA cleaving properties (at least in vitro), but this is at the expense of cytotoxic activity. The reduced ability of the conjugates to interfere with topoisomerase II, compared with the metal-free anthraquinone derivatives, may account for their reduced cytotoxic potential. Other factors such as changes in the redox potential, inappropriate metabolic activation, cellular distribution, and/or cellular uptake may also be involved. The nature of the metal chelated by the salen moiety influences the capacity of a

particular anthraquinone–salen conjugate to bind to DNA and cleave it. The selectivity for binding to GC-rich sequences conferred by the anthraquinone moiety is retained with the nickel complexes, but not with the copper complexes. Despite the observation that the conjugates **22** and **24** exhibit moderate cytotoxic activity and inhibit topoisomerase II, anthraquinone–salen•Cu^{II} hybrid molecules apparently are not very promising as antitumour agents, but they might turn out to be interesting as DNA cleavers. Salen•Cu^{II} complexes equipped with an intercalating chromophore such as compounds **20** and **22**, which efficiently cleave double-stranded DNA irrespective of its primary nucleotide sequence, might be used as probes for investigating DNA structure and/or the sequence-specificity of protein–DNA interactions. Studies currently under way will determine whether anthraquinone–salen conjugates complexed with various divalent metals such as Cu^{II} or Mn^{II} can be employed as footprinting agents as is the case with other metal chelates attached to an intercalating drug (e.g., methidiumpropyl–EDTA•Fe^{II}, acridine–EDTA•Fe^{II}).

Experimental

Synthesis

The purity of all compounds was assessed by TLC, ^1H and ^{13}C NMR, and MS. Kieselgel 60 (004–0063 mesh, Merck) was used for flash chromatography. TLC was carried out using Silica Gel 60F-254 (Merck, 0.25 mm thick) precoated UV sensitive plates. Spots were visualized by inspection under visible light or UV at 254 nm. Melting points were determined in a Thermopan hot plate microscope and are uncorrected. ^1H NMR spectra were recorded on a Bruker AM 300 WB. Chemical shifts were measured from tetramethylsilane as an internal reference and are given in δ ppm units; coupling constants (*J*) are given in Hz. IR spectra were measured with a Perkin–Elmer 1310 spectrophotometer using KBr pellets, and only the principal sharp peaks are given. FABMS were determined on a Kratos MS-50 RF mass spectrometer arranged in EBE geometry. Samples were bombarded using a beam of xenon with a kinetic energy of 7 keV. The mass spectrometer was operated at 8 kV accelerating voltage with a mass resolution of 3000. IE mass spectra were determined on a quadrupolar spectrometer Ribermag R10–10 with a kinetic energy of 70 eV. Optical rotations were measured with a Perkin–Elmer polarimeter (Model 241).

***Nα*-Z-*Nε*-Boc-L-lysinamide (2).** A solution of *Nα*-Z-*Nε*-Boc-L-lysine (**1**, 2 g, 5.26 mmol), NEt_3 (0.74 mL, 5.31 mmol) and ethyl choroformate (0.55 mL, 5.75 mmol) in dry THF (30 mL) was stirred under argon at −10 °C for 45 min. Ammonia-saturated THF (30 mL) was added and the mixture was stirred for 1 h at −10 °C and then overnight at room temperature. The solvent was removed by distillation under reduced pressure and the residue was dissolved in EtOAc (150 mL). The organic layer was washed with 1 N Na_2CO_3 (100 mL) and water (100 mL). After drying over Na_2SO_4 and filtration, the solvent was evaporated under reduced pressure. Compound **2** was obtained as a white solid (1.7 g, 85%): mp 141–142 °C; $[\alpha]_D^{25}$ −1.75 (*c* 4, MeOH); IR (KBr): ν 3370–3310, 3200, 2965, 1680, 1655, 1535 cm^{-1}; EI-MS: 380 $(M+1)^+$; R_f (MeOH:$CHCl_3$, 20:80), 0.74; ^1H NMR ($CDCl_3$): δ 1.4 (s, 9H), 1.47 (m, 4H), 1.62 (m, 2H), 3.06 (m, 2H), 4.15 (m, 1H), 5.10 (s, 2H), 5.75 (m, 2H), 6.40 (m, 2H); ^{13}C NMR ($CDCl_3$): δ 22.6 (CH_2), 28.40 (CH_3), 29.43 (CH_2), 40.02 (CH_2), 44.38 (CH_2), 64.64 (CH_2), 77.32 (CH), 78.96 (Cq), 126.88 (CH), 127.23 (CH), 141.41(Cq), 156.33 (Cq). Anal. calcd for $C_{19}H_{29}N_3O_5$: C, 60.16; H, 7.65; N, 11.08. Found: C, 59.98; H, 7.62; N, 11.07.

(*S*)-1-Cyano-*N*-[benzyloxycarbonyl]-*N'*-[*tert*-butyloxycarbonyl]-1,5-diaminopentane (3). A solution of **2** (1.6 g, 4.21 mmol), NEt_3 (1.4 mL, 10.04 mmol) in THF (20 mL) was stirred under argon at 0 °C. Trifluoroacetic anhydride (0.7 mL, 4.95 mmol) was added dropwise and the mixture was stirred for 1 h at 0 °C, then overnight at room temperature. The solvent was removed by distillation under reduced pressure and the residue was dissolved in Et_2O (150 mL). The organic layer was washed in turn with 0.1 N HCl (100 mL), 0.1 N NaOH (100 mL), and water (100 mL). After drying over Na_2SO_4 and filtration, the solvent was evaporated under reduced pressure. Compound **3** was obtained as a white solid (1.26 g, 83%): mp 81–82 °C; $[\alpha]_D^{25}$ −27 (*c* 4, MeOH); IR (KBr): ν 3370, 2965, 1715, 1690, 1540 cm^{-1}; EI-MS: 362 $(M+1)^+$; R_f (MeOH:$CHCl_3$, 20:80), 0.85; ^1H NMR ($CDCl_3$): δ 1.38 (m, 11H), 1.47 (m, 4H), 3.10 (m, 2H), 4.53 (m, 1H), 5.14 (s, 2H), 5.76 (m, 2H), 7.34 (m, 5H); ^{13}C NMR ($CDCl_3$): δ 21.27 (CH_2), 28.37 (CH_3), 29.38 (CH_2), 32.11 (CH_2), 39.42 (CH_2), 42.65 (CH), 67.63 (CH2), 79.49 (Cq), 118.61 (Cq), 128.35 (CH), 128.59 (CH), 135.66 (Cq), 156.38 (Cq), 155.39 (Cq). Anal calcd for $C_{19}H_{27}N_3O_4$: C, 63.16; H, 7.48; N, 11.63. Found: C, 63.28; H, 7.46; N, 11.59.

(*S*)-6-(*tert*-Butyloxycarbonyl)-1,2,6-triaminohexane (4). A solution of **3** (500 mg, 1.38 mmol) containing Raney nickel (1 g) in ammonia saturated MeOH (20 mL) was stirred under 50 hydrogen atmosphere at 50 °C for 24 h. The reaction mixture was filtered and the solvent was evaporated under reduced pressure to give compound **4** as a pink solid (0.3 g, 93%): mp 46–48 °C; IR (KBr): ν 3200–3600, 2800–2900, 1690, 1540 cm^{-1}; EISM 232 $(M+1)^+$; R_f (MeOH:$CHCl_3$, 40:60), 0.15; ^1H NMR ($CDCl_3$): δ 1.3–1.6 (m, 15H), 1.88 (m, 2H), 2.7–3.1 (m, 3H), 5.23 (m, 4H), 7.32 (m, 1H); ^{13}C NMR ($CDCl_3$): δ 22.28 (CH_2), 23.74 (CH_2), 28.43 (CH_3), 29.43 (CH_2), 32.66 (CH_2), 39.78 (CH_2), 50.50 (CH), 79.04 (Cq), 156.25 (Cq); Anal calcd for $C_{11}H_{25}N_3O_2$: C, 57.14; H, 10.82; N, 18.18. Found: C, 57.25; H, 10.78; N, 18.13.

(*S*)-*N,N'*-(Bis-salicylidene)-6-(*tert*-butyloxycarbonyl)-1,2,6-triaminohexane copper complex (5). A solution of **4** (0.3 g, 1.3 mmol), salicylaldehyde (0.65 mL, 6.10 mmol) in dry EtOH (25 mL) and copper(II) acetate

monohydrate (0.57 g, 3.05 mmol in 5 mL of water) was refluxed under argon for 4 h. The solvent was distilled under reduced pressure and the residue was triturated with CH_2Cl_2. After filtration of the copper salts, the solvent was evaporated under reduced pressure. Compound **5** was recrystallized from ethanol:water (0.36 g, 55%): mp 135–136 °C; $[\alpha]_D^{25}$ −25 (*c* 0.04, MeOH); IR (KBr): ν 3400, 3370, 2950, 1715, 1615 cm^{-1}; MS (FAB+): 501$(M+1)^+$, 1002 $(2\times(M+1))^+$; R_f (MeOH), 0.00; ESR: $A_{||}$ 192.5 G, $g_{||}$ 2.22. Anal. calcd for $C_{25}H_{31}N_3O_4Cu$: C, 59.94; H, 6.19; N, 8.39. Found: C, 59.76; H, 6.17; N, 8.42.

(*S*)-*N,N'*-(Bis-salicylidene)-1,2,6-triaminohexane trifluoroacetic acid copper complex (6). Trifluoroacetic acid (1.01 mL, 11.41 mmol) was added dropwise at room temperature to a solution of **5** (0.1 g, 0.19 mmol) in CH_2Cl_2 (15 mL) containing anisole (5 μL, 0.04 μmol). The resulting mixture was stirred for 30 min and then evaporated to dryness. The residue was dissolved in EtOH and the solvent evaporated to dryness several times. Compound **6** was recrystallized from EtOH as a green solid (60 mg, 61%): mp 158–160 °C; $[\alpha]_D^{25}$ −8 (*c* 0.05, DMSO); IR (KBr): ν 3200, 1620 cm^{-1}; MS (FAB+): 401 $(M-CF_3CO_2^-)^+$, 802 $(2\times(M))^+$; R_f (MeOH), 0.00; ESR: $A_{||}$ 191.25 G, $g_{||}$ 2.23. Anal. calcd for $C_{22}H_{24}N_3O_4F_3Cu$: C, 51.31; H, 4.66; N, 8.16. Found: C, 51.20; H, 4.64; N, 8.13.

(*S*)-*N,N'*-(Bis-salicylidene)-1,2,6-triaminohexane nickel complex (7). A solution of **4** (0.4 g, 1.72 mmol), salicylaldehyde (0.65 mL, 6.10 mmol) in dry EtOH (25 mL) and nickel(II) acetate tetrahydrate (1.6 g, 6.42 mmol) was refluxed under argon for 3 h. The solvent was evaporated under reduced pressure and the crude material was recrystallized from Et_2O to give compound **7** as an orange solid (0.35 g, 41%): mp 135–137 °C; $[\alpha]_D^{25}$ −52 (*c* 0.05, DMSO); IR (KBr): ν 3300, 2900, 1715, 1640 cm^{-1}; MS (FAB+): 496 $(M+1)^+$, 991 $(2\times(M)+1)^+$; R_f (MeOH), 0.00; ^1H NMR (acetone-d_6): δ 1.36 (m, 11H), 1.55 (m, 2H), 1.87 (m, 2H), 3.03 (m, 2H), 3.20 (m, 1H), 3.39 (m, 1H), 3.76 (m, 1H), 5.96 (m, 1H), 6.48 (m, 2H), 6.75 (m, 2H), 7.16 (m, 4H), 7.80 (s, 2H); ^{13}C NMR (acetone-d_6): δ 28.09 (CH_2), 34.12 (CH_2, CH_3), 40.53 (CH_2), 45.03 (CH_2), 67.60 (CH_2), 73.46 (CH), 85.89 (Cq), 120.59 (CH), 125.58 (CH), 126.11 (Cq), 137.71 (CH), 137.98 (CH), 138.58 (CH), 167.00 (CH), 168.00 (CH), 170.38 (Cq). Anal. calcd for $C_{25}H_{31}N_3O_4Ni$: C, 60.52; H, 6.25; N, 8.47. Found: C, 60.64; H, 6.23; N, 8.51.

(*S*)-*N,N'*-(Bis-salicylidene)-1,2,6-triaminohexane trifluoroacetic acid nickel complex (8). Trifluoroacetic acid (1.01 mL, 11.41 mmol) was added dropwise at room temperature to a solution of **7** (0.2 g, 0.4 mmol) in CH_2Cl_2 (15 mL) containing anisole (5 μL, 0.04 μmol). The mixture was stirred for 30 min and then evaporated to dryness. The residue was dissolved in EtOH and the solvent evaporated to dryness several times. Compound **8** was crystallized as an orange hygroscopic solid from EtOH (0.16 g, 78%): mp 150–152 °C; IR (KBr): ν 3200, 1640 cm^{-1}; MS (FAB+): 396 $(M-CF_3CO_2^-)^+$; $[\alpha]_D^{25}$ −49 (*c* 0.05, DMSO); R_f (MeOH), 0.00; ^1H NMR (DMSO-d_6): δ 1.35 (m, 2H), 1.57 (m, 2H), 1.69 (m, 2H), 2.77 (m, 2H), 2.88 (m, 1H), 3.09 (m, 1H), 3.66 (m, 1H), 6.49 (m, 2H), 6.67 (m, 2H), 7.15 (m, 2H), 7.28 (m, 2H), 7.72 (s, 3H), 7.84 (s, 2H); ^{13}C NMR (DMSO-d_6): δ 22.09 (CH_2), 26.40 (CH_2), 26.66 (CH_2), 34.58 (CH_2), 61.88 (CH_2), 67.26 (CH), 119.66 (CH), 120.13 (Cq), 120.34 (Cq), 132.68 (CH), 133.49 (CH), 162.21 (CH), 163.06 (CH), 163.80 (Cq). Anal calcd for $C_{22}H_{24}N_3O_4F_3Ni$: C, 51.79; H, 4.71; N, 8.24. Found: C, 51.68; H, 4.72; N, 8.26.

1,4-Bis[Boc-aminoethylamino]anthracene-9,10-dione (11) and 1-[(Boc-aminoethyl)amino]-4-hydroxyanthracene-9,10-dione (12). The mono- and disubstituted anthraquinones **11** and **12** were synthesized from leucoquinizarine **9** (0.8 g, 3.50 mmol) and Boc^t-ethylenediamine (5.28 g, 33 mmol) according to the procedure previously described.[42] Compounds **11** and **12** were purified by flash chromatography on a silica gel.

Compound 11. Blue solid (1.02 g, 55%): mp 214–216 °C; IR (KBr) ν 3400, 2980, 1720, 1620 cm^{-1}; MS (FAB+): 525 $(M+1)^+$; R_f (CH_2Cl_2), 0.26; ^1H NMR (DMSO-d_6): δ 1.34 (s, 18 H) 3.17 (t, J=5.8 Hz, 4H), 3.47 (t, J = 5.9 Hz, 4H), 7.03 (m, 2H), 7.52 (s, 2H), 7.77 (d, J=5.8 Hz, 2H), 8.21 (d, J=5.8 Hz, 2H), 10.84 (s, 2H); ^{13}C NMR (DMSO-d_6): δ 28.21 (CH_3), 39.78 (CH_2), 41.55 (CH_2), 77.79 (Cq), 108.67 (Cq), 124.34 (CH), 125.63 (CH), 132.29 (CH), 133.83 (CH), 146.03 (Cq), 155.81 (Cq), 180.70 (Cq). Anal. calcd for $C_{28}H_{36}N_4O_6$: C, 64.12; H, 6.97; N, 10.68. Found: C, 63.99; H, 6.99; N, 10.65.

Compound 12. Violet solid (0.31 g, 23%): mp 167–168 °C; IR (KBr): ν 3400, 2990, 1710, 1620 cm^{-1}; MS (FAB+): 383 $(M+1)^+$; R_f (CH_2Cl_2), 0.85; ^1H NMR (DMSO-d_6): δ 1.34 (s, 9H), 3.18 (t, J=5.9 Hz, 2H), 3.47 (t, J=6.0 Hz, 2H), 7.04 (m, 1H), 7.29 (d, J=9.6 Hz, 1H), 7.32 (d, J=9.7 Hz, 2H), 7.85 (m, 2H), 8.20 (m, 2H), 10.26 (s, 1H), 10.43 (s, 1H); ^{13}C NMR (DMSO-d_6): δ 28.18 (CH_3), 39.67 (CH_2), 41.57 (CH_2), 77.80 (Cq), 107.43 (Cq), 112.76 (Cq), 125.17 (CH), 125.82 (CH), 126.14 (CH), 128.45 (Cq), 131.74 (Cq), 132.75 (CH), 134.52 (CH), 134.60 (Cq), 147.27 (Cq), 155.80 (Cq), 180.51 (Cq), 186.56 (Cq). Anal. calcd for $C_{21}H_{22}N_2O_5$: C, 65.97; H, 5.76; N, 7.33. Found: C, 65.77; H, 5.76; N, 7.85.

1,4-Bis[Boc-aminoethylamino]-5,8-dihydroxyanthracene-9,10-dione (13). Reaction of 5,8-dihydroxyleucoquinizarine (0.94 g, 3.43 mmol) with freshly distilled Boc^t-ethylenediamine (4.7 g, 29.3 mmol) under the conditions previously described[42] afforded the anthraquinone **13**, which was obtained as a blue solid after purification by flash chromatography (1.02 g, 55%): mp 187–188 °C; IR (KBr): ν 3300, 2950, 1690, 1620 cm^{-1}; MS (FAB+): 557 $(M+1)^+$; R_f (CH_2Cl_2:AcOEt, 50:50), 0.35; ^1H NMR (DMSO-d_6): δ 1.35 (s, 18 H), 3.02 (t, J=5.9 Hz, 4H), 3.54 (t, J=5.8 Hz, 4H), 7.07 (m, 2H), 7.14 (s, 2H), 7.58 (s, 2H), 10.50 (s, 2H), 10.61 (s, 2H); ^{13}C NMR (DMSO-d_6): δ 38.38 (CH_3), 45.54 (CH_2), 47.18 (CH_2), 77.46 (Cq), 83.06 (Cq), 112.61

(Cq), 120.16 (Cq), 129.54 (CH), 130.68 (CH), 152.45 (Cq), 159.78 (Cq), 188.56 (Cq). Anal. calcd for $C_{28}H_{36}N_4O_8$: C, 60.43; H, 6.47; N, 10.07. Found: C, 60.30; H, 6.46; N, 10.10.

1,4-[Aminoethylamino]anthracene-9,10-dione bis-trifluoroacetic acid (14). Trifluoroacetic acid (10 mL) was added dropwise to a well stirred solution of **6** (0.9 g, 1.71 mmol) in 10 mL of CH_2Cl_2. The resultant mixture was maintained at room temperature for 45 min prior to evaporation of the solvent under vacuo. The crude residue was treated with ethanol to obtain compound **14** as a blue solid (0.94 g, 100%): mp 203–205 °C; IR (KBr): ν 3300, 2900, 1690 cm^{-1}; MS (FAB+): 326 $(M)^{2+}$; R_f (MeOH), 0.00; ^1H NMR (DMSO-d_6): δ 3.08 (m, 4H), 3.73 (m, 4H), 7.51 (s, 2H), 7.83 (d, $J=5.8$ Hz, 2H), 8.04 (s, 6H), 8.24 (d, $J=5.8$ Hz, 2H), 10.64 (s, 2H); ^{13}C NMR (DMSO-d_6): δ 38.36 (CH_2), 39.58 (CH_2), 109.68 (Cq), 123.99 (CH), 125.77 (CH), 132.75 (CH), 133.68 (Cq), 145.34 (Cq), 181.56 (Cq). Anal. calcd for $C_{22}H_{22}N_4O_6F_6$: C, 47.82; H, 3.98; N, 10.14. Found: C, 47.67; H, 3.97; N, 10.11.

1-[Aminoethylamino]-4-hydroxyanthracene-9,10-dione trifluoroacetic acid (15). Treatment of **12** (0.30 g, 0.78 mmol) with trifluoroacetic acid under the same conditions as described for **14** afforded the mono-substituted anthraquinone **15** as a violet solid (0.307 g, 100%): mp 122–124 °C; IR (KBr): ν 3400, 2900, 1680 cm^{-1}; MS (FAB+): 283 $(M-CF_3CO_2^-)^+$; R_f (MeOH), 0.00; ^1H NMR (DMSO-d_6): δ 3.04 (m, 2H), 3.66 (m, 2H), 7.30 (d, $J=9.6$ Hz, 1H), 7.49 (d, $J=9.7$ Hz, 1H), 7.87 (m, 2H), 8.03 (s, 3H), 8.22 (d, $J=6.9$ Hz, 2H), 10.14 (m, 1H); ^{13}C NMR (DMSO-d_6): δ 38.10 (CH_2), 39.56 (CH_2), 77.80 (Cq), 108.36 (Cq), 113.05 (Cq), 124.99 (CH), 125.99 (CH), 126.29 (CH), 128.54 (CH), 131.83 (Cq), 133.13 (CH), 134.50 (Cq), 134.77 (CH), 146.67 (Cq), 156.09 (Cq), 181.16 (Cq), 186.84 (Cq). Anal. calcd for $C_{18}H_{15}N_2O_5F_3$: C, 54.55; H, 3.81; N, 7.07. Found: C, 54.69; H, 3.85; N, 7.22.

1,4-Bis[aminoethylamino]-5,8-dihydroxyanthracene-9,10-dione bistrifluoroacetic acid (16). The Boc-protecting group of compound **12** was cleaved with trifluoroacetic acid as described for **14**. Blue solid (0.31 g, 100%): mp >350 °C; IR (KBr) ν 3400, 2900, 1680 cm^{-1}; MS (FAB+): 358 $(M)^{2+}$; R_f (MeOH), 0.00; ^1H NMR (DMSO-d_6): δ 3.07 (m, 4H), 3.75 (m, 4H), 7.20 (s, 2H), 7.55 (s, 2H), 8.01 (s, 6H), 10.42 (s, 2H), 13.46 (s, 2H); ^{13}C NMR (DMSO-d_6): δ 38.26 (CH_2), 39.75 (CH_2), 108.43 (Cq), 114.55 (Cq), 124.73 (CH), 124.95 (CH), 146.34 (Cq), 154.76 (Cq), 184.23 (Cq). Anal. calcd for $C_{22}H_{22}N_4O_8F_6$: C, 45.20; H, 3.76; N, 9.59. Found: C, 45.13; H, 3.77; N, 9.61.

1,4-Bis[2-(succinylaminoethyl)amino]anthracene-9,10-dione (17). Succinic anhydride (0.73 g, 6.18 mmol) and 10 mL of pyridine were added dropwise to a solution of **14** (0.96 g, 1.73 mmol) in DMF (15 mL). The mixture was stirred at reflux for 3 h. The solvent was evaporated and the crude residue was dissolved in 1 N HCl (80 mL) and stirred for 15 min. Compound **17** was collected by filtration and successively washed with EtOAc (50 mL) and Et_2O (50 mL, 0.86 g, 94%): mp 179–180 °C; IR (KBr) ν 3400, 2900, 1715, 1640 cm^{-1}; MS (FAB+): 525 $(M+1)^+$; R_f (MeOH), 0.83; ^1H NMR (DMSO-d_6): δ 2.33 (t, $J=6.9$ Hz, 4H), 2.43 (t, $J=7.4$ Hz, 4H), 3.28 (t, $J=5.6$ Hz, 4H), 3.49 (t, $J=5.4$ Hz, 4H), 7.55 (s, 2H), 7.77 (d, $J=5.8$ Hz, 2H), 8.15 (m, 2H), 8.22 (d, $J=5.8$ Hz, 2H), 10.80 (s, 2H); ^{13}C NMR (DMSO-d_6): δ 29.09 (CH_2), 29.98 (CH_2), 38.77 (CH_2), 41.27 (CH_2), 108.69 (Cq), 124.41 (CH), 125.67 (CH), 132.31 (CH), 133.81 (Cq), 145.98 (Cq), 171.57 (Cq), 173.83 (Cq), 181.13 (Cq). Anal. calcd for $C_{26}H_{28}N_4O_8$: C, 59.54; H, 5.38; N, 10.68. Found: C, 59.72; H, 5.39; N, 10.82.

1-[2-(Succinylaminoethyl)amino]-4-hydroxyanthracene-9,10-dione (18). Succinic anhydride (0.10 g, 0.84 mmol) and 1.5 mL of pyridine were added dropwise to a solution of **15** (0.15 g, 0.37 mmol) in DMF (15 mL). The solution was stirred at reflux for 3 h prior to evaporation of the solvent. The resulting crude residue was dissolved in 10% citric acid (50 mL) and stirred for 15 min. Compound **18** was collected by filtration and successively washed with AcOEt (10 mL) and Et_2O (10 mL; 0.73 mg, 51%): mp 181–183 °C; IR (KBr): ν 3400, 2900, 1740, 1640 cm^{-1}; MS (FAB+): 383 $(M+1)^+$; R_f (MeOH), 0.72; ^1H NMR (DMSO-d_6): δ 2.29 (t, $J=6.3$ Hz, 4H), 2.42 (t, $J=6.5$ Hz, 2H), 3.29 (m, 2H), 3.49 (m, 2H), 7.36 (d, $J=9.6$ Hz, 1H), 7.57 (d, $J=9.6$ Hz, 1H), 7.90 (m, 2H), 8.15 (s, 1H), 8.25 (d, $J=7.1$ Hz, 2H), 10.30 (s, 1H), 10.40 (s, 1H), 12.10 (s, 1H); ^{13}C NMR (DMSO-d_6): δ 29.07 (CH_2), 29.97 (CH_2), 38.58 (CH_2), 41.33 (CH_2), 107.62 (Cq), 112.91 (Cq), 125.46 (CH), 125.99 (CH), 126.33 (CH), 128.72 (Cq), 131.89 (Cq), 132.98 (CH), 134.73 (CH), 147.39 (Cq), 156.18 (Cq), 171.58 (Cq), 173.81 (Cq), 180.81 (Cq), 186.79 (Cq). Anal. calcd for $C_{20}H_{18}N_2O_6$: C, 62.82; H, 4.71; N, 7.33. Found: C, 62.94; H, 4.72; N, 7.31.

1,4-Bis[2-(succinylaminoethyl)amino]-5,8-dihydroxyanthracene-9,10-dione (19). Succinic anhydride (0.50 g, 4.23 mmol) and 10 mL of pyridine were added dropwise to a solution of **16** (0.86 g, 1.47 mmol) in 30 mL of DMF. The mixture was stirred at reflux for 2 h. The solvent was evaporated and the crude residue was dissolved in 1 N HCl (80 mL) and stirred for 15 min. A blue solid was collected by filtration and successively washed with AcOEt (50 mL) and Et_2O (50 mL). After drying, compound **19** was obtained as a blue solid (0.65 g, 80%): mp 241–243 °C; IR (KBr): ν 3400, 2900, 1700, 1640 cm^{-1}; MS (FAB+): 557 $(M+1)^+$; R_f (MeOH), 0.90; ^1H NMR (DMSO-d_6): δ 2.32 (t, $J=6.3$ Hz, 4H), 2.42 (t, $J=6.0$ Hz, 4H), 3.30 (t, $J=5.8$ Hz, 4H), 3.53 (t, $J=5.9$ Hz, 4H), 7.12 (s, 2H), 7.59 (s, 2H), 8.16 (m, 2H), 10.54 (s, 4H); ^{13}C NMR (DMSO-d_6): δ 29.07 (CH_2), 29.98 (CH_2), 43.82 (CH_2), 46.74 (CH_2), 107.34 (Cq), 114.79 (Cq), 124.09 (CH), 124.92 (CH), 146.85 (Cq), 154.52 (Cq), 171.64 (Cq), 173.82 (Cq), 181.19 (Cq). Anal. calcd for $C_{26}H_{28}N_4O_{10}$: C, 56.11; H, 5.03; N, 10.07. Found: C, 56.22; H, 5.05; N, 10.11.

(*S,S*)-1,4-Bis[[2-[1-[4-(*N,N'*-(bis-salicylidenamino) ethyl)aminobutyl]succinyl]aminoethyl]diamino] anthracene-9,10-dione bis-Cu^{II} complex (20). A solution of **17** (20 mg, 38.16 μmol), dicyclohexylcarbodiimide (DCC, 17.3 mg, 83.84 μmol) and *N*-hydroxybenzotriazole (HOBt, 11.34 mg, 83.91 μmol) in dry DMF (50 mL) was stirred at 0 °C. After 1 h, a solution of the salen•Cu^{II} complex **6** (41.19 mg, 80.05 μmol) and Et_3N (11 μL, 82.73 μmol) was added. The mixture was stirred at room temperature for 4 days. The solvent was removed in vacuo and the crude material was successively washed with $CHCl_3$ (20 mL), 10% citric acid (25 mL) and methanol (20 mL), and finally by Et_2O (30 mL). The anthraquinone–salen•Cu^{II} conjugate **20** was obtained as a black solid (18 mg, 37%): mp 174–176 °C; IR (KBr): ν 3400, 2920, 1630 cm^{-1}; MS (FAB+): 1290 $(M+1)^+$; R_f (MeOH), 0.00; ESR: $A_{||}$ 194 G, $g_{||}$ 2.22. Anal. calcd for $C_{66}H_{70}N_{10}O_{10}Cu_2$: C, 61.44; H, 5.43; N, 10.86. Found: C, 61.25; H, 5.42; N, 10.82.

(*S*)-1-[[2-[1-[4-(*N,N'*-(Bis-salicylidenamino)ethyl) aminobutyl]succinyl]aminoethyl]amino]-4-hydroxy-anthracene-9,10-dione Cu^{II} complex (21). Compound **21** was synthesized by coupling the acid **18** and the amine **6** using DCC–HOBt as described for **20**. (42.1 mg, 79%): mp 138–140 °C; $[\alpha]_D^{25}$ <1 (c 5×10^{-4}, DMSO); IR (KBr): ν 3400, 2920, 1630, 1625 cm^{-1}; MS (FAB+): 766 $(M+1)^+$; R_f (MeOH), 0.10; ESR: $A_{||}$ 194.2 G, $g_{||}$ 2.23. Anal. calcd for $C_{40}H_{39}N_5O_7Cu$: C, 62.78; H, 5.10; N, 9.15. Found: C, 62.65; H, 5.08; N, 9.11.

(*S,S*)-1,4-Bis[[2-[1-[4-((*N,N'*-bis-salicylidenamino) ethyl)aminobutyl]succinyl]aminoethyl]diamino]-5,8-dihydroxyanthracene-9,10-dione bis-Cu^{II} complex (22). Compound **22** was synthesized by coupling the acid **19** and the amine **6** using DCC–HOBt as described for **20** (43 mg, 86%): mp 190–192 °C; IR (KBr): ν 3400, 2920, 1630, 1625 cm^{-1}; MS (FAB+): 1322 $(M+1)^+$; R_f (MeOH), 0.00; ESR: $A_{||}$ 197.5 G, $g_{||}$ 2.22. Anal. calcd for $C_{66}H_{70}N_{10}O_{12}Cu_2$: C, 59.95; H, 5.30; N, 10.60. Found: C, 59.83; H, 5.32; N, 10.62.

(*S*)-1-[[2-[1-[4-((*N,N'*-bis-salicylidenamino)ethyl) aminobutyl]succinyl]aminoethyl]amino]-4-hydroxy-anthracene-9,10-dione Ni^{II} complex (23). The monosubstituted anthraquinone–salen•Ni^{II} conjugate **23** was prepared by coupling the amine **8** with the acid **18** as described for **21**. Violet solid (36.7 mg, 49%): mp 182–183 °C; $[\alpha]_D^{25}$ <1 (c 5×10^{-4}, DMSO); IR (KBr): ν 3200, 2900, 1625 cm^{-1}; MS (FAB+): 760 $(M+1)^+$; R_f (MeOH), 0.20; ^{1}H NMR (DMSO-d_6): δ 1.00–1.70 (m, 6H), 2.27–2.35 (m, 4H), 3.00–3.60 (m, 9H), 5.58 (m, 1H), 6.48 (m, 2H), 6.66 (s, 2H), 7.11–7.76 (m, 8H), 8.22 (m, 3H), 10.28 (m, 2H); ^{13}C NMR (DMSO-d_6): δ 28.81 (CH_2), 30.77 (CH_2), 33.34 (CH_2), 34.84 (CH_2), 38.09 (CH_2), 40.42 (CH_2), 41.39 (CH_2), 47.50 (CH_2), 61.85 (CH), 67.42(CH_2), 107.62 (Cq), 112.95 (Cq), 119.77 (CH), 120.21 (CH), 120.41 (CH), 125.52 (CH), 126.03 (CH), 128.04 (Cq), 128.74 (Cq), 131.93 (Cq), 132.73 (Cq), 133.01 (CH), 133.59 (Cq), 134.76 (CH), 147.41 (Cq), 156.20 (Cq), 156.62 (Cq), 162.16 (CH), 163.12 (CH), 164.11 (Cq), 171.13 (Cq), 172.05 (Cq), 180.88 (Cq), 186.84 (Cq). Anal. calcd for $C_{40}H_{39}N_5O_7Ni$: C, 63.18; H, 5.13; N, 9.21. Found: C, 63.05; H, 5.11; N, 9.25.

(*S,S*)-1,4-[[2-[1-[4-((*N,N'*-Bis-salicylidenamino)ethyl) aminobutyl]succinyl]aminoethyl]diamino]-5,8-dihydroxyanthracene-9,10-dione bis-Ni^{II} complex (24). Compounds **19** (25.5 mg, 45.78 μmol) and **8** (63.1 mg, 123.96 μmol) were coupled as described for **20**. Green solid (33 mg, 56%): mp >350 °C; IR (KBr): ν 3400, 2950, 1640, 1620 cm^{-1}; MS (FAB+) 1312 $(M+1)^+$; R_f (MeOH), 0.00; ^{1}H NMR (DMSO-d_6): δ 1.30–1.80 (m, 12H), 2.20–2.30 (m, 8H), 2.75 (m, 2H), 3.90–3.60 (m, 16H), 6.48 (m, 2H), 6.50 (m, 4H), 6.60 (m, 4H), 7.00–7.20 (m, 10H), 7.60 (m, 2H), 7.80 (m, 4H), 8.12 (5m, 4H), 10.54 (m, 4H); ^{13}C NMR (DMSO-d_6): δ 23.11 (CH_2), 32.20 (CH_2), 35.71 (CH_2), 37.37 (CH_2), 38.96 (CH_2), 36.61 (CH_2), 42.55 (CH_2), 43.16 (CH_2), 68.31 (CH), 115.17 (CH), 120.68 (CH), 125.27 (CH), 126.38 (CH), 129.28 (CH), 133.67 (CH), 134.50 (CH), 157.35 (CH) 163.11 (CH), 164.01 (CH). Anal. calcd for $C_{66}H_{70}N_{10}O_{12}Ni_2$: C, 60.79; H, 5.33; N, 10.67. Found: C, 60.74; H, 5.31; N, 10.64.

DNase I footprinting

The restriction fragments used in the footprinting and DNA cleavage experiments were purified and labelled using standard methods.[61] DNase I footprinting experiments were performed essentially according to the protocol recently described.[62] Briefly, reactions were conducted in a total volume of 10 μL. Samples (3 μL) of the labelled DNA fragment were incubated with 5 μL of buffer solution containing the desired drug concentration. After 30–60 min incubation at 37 °C to ensure equilibration, the digestion was initiated by adding 2 μL of a DNase I solution so as to yield a final enzyme concentration of ca. 0.01 unit/mL. After 3 min, the digestion was stopped by addition of 3 μL of an 80% formamide solution containing tracking dyes. Samples were heated at 90 °C for 4 min and chilled in ice for 4 min prior to electrophoresis. Electrophoresis, autoradiography and quantitation by storage phosphor imaging were performed as previously described.[61]

DNA cleavage

Each reaction mixture contained 4 μL of supercoiled pUC12 DNA (3 μg), 5 μL of drug (2 to 400 μM) and 1 μL of MPA (25 mM) to initiate the reaction. After 2 h incubation at 37 °C, 1 μL of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in H_2O) was added to each tube and the solution was loaded on to a 0.9% agarose gel. Electrophoresis was carried out for about 2 h at 100 V in TBE buffer (89 mM tris-borate pH 8.3, 1 mM EDTA). Gels were stained with ethidium bromide (1μg/mL) then destained for 30 min in water prior to being photographed under UV light.

ESR

ESR measurements were recorded on a Varian E-109 X-band spectrometer with a dual cavity operating in the TE 104 mode for analyzing the copper complexes. A 100 kHz high frequency modulation with a maximum amplitude of 8 Gauss was used with a 10 mW microwave power and g values were determined from the Varian 'strong pitch', $g=2.0028$. 10 mM solutions of salen•Cu^{II} complexes in Me_2SO were dispensed into a 4 mm diameter cylindrical quartz tube. ESR analyses were conducted at 77 K on glycerol glasses. For the spin trapping experiments, we used a cavity operating in the TM 110 mode with a maximum modulation amplitude of 1 Gauss and a 100 kHz high-frequency modulation with 10 mW microwave power. The solutions were examined in flat quartz cells inserted in an E-238 cavity. The spin-trapping agent 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO, purchased from Sigma Chem. Co.) was used at a concentration of 7 mg/mL. The post-DMPO time was 8 min.

Topoisomerase I and II DNA cleavage reaction

Topoisomerase I and topoisomerase II were purified from calf thymus using an adaptation of procedures described previously.[63] The cleavage reaction mixture contained 20 mM Tris HCl pH 7.4, 60 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol (plus 10 mM $MgCl_2$ and 1 mM ATP for topoisomerase II), 2×10^{-4} dpm of α-^{32}P-pBR322 DNA and the indicated drug concentrations. The reaction was initiated by the addition of topoisomerase I or II (20 units in 20 μL reaction volume) and allowed to proceed for 10 min at 37 °C. Reactions were stopped by adding SDS to a final concentration of 0.25% and proteinase K to 250 μg/mL, followed by incubation for 30 min at 50 °C. For topoisomerase I, samples were denatured by the addition of 10 μL denaturing loading buffer consisting of 0.45 M NaOH, 30 mM EDTA, 15% (w/v) sucrose, 0.1% bromocresol green prior to loading onto a 1% agarose gel in TBE buffer (89 mM Tris base, 89 mM boric acid, 2.5 mM Na_2 EDTA, pH 8.3) containing 0.1% SDS. Electrophoresis was maintained at 2 V/cm for 18 h.

Cell cultures and growth inhibition assay

The KB human carcinoma cell line was obtained from the ATCC (Rockville, Maryland). Cells were grown as monolayers in Dulbecco's Modified Eagle Medium containing 2 mM glutamine, 200 IU/mL penicillin, 200 μg/mL streptomycin, 50 μg/mL gentamicin, and 50 μg/mL nystatin supplemented with 10% (v/v) heat inactivated fetal calf serum at 37 °C in a humidified atmosphere containing 5% CO_2. Experiments were carried out with exponentially growing cells as previously described.[64] Briefly, 2×10^{-4} cells/mL were seeded in 96-well microculture plates with various drug concentrations (semi-log dilutions). After a 96 h incubation at 37 °C, cells were incubated with 0.02% neutral red for 16 h, then washed and lysed with 1% SDS. The incorporation of the dye, which reflects cellular growth and viability, was evaluated by measuring the optical density at 540 nm for each well using a Titertek multiwell spectrophotometer. Each point was measured in quadruplicate and results were expressed as percent of cell growth inhibition determined relative to that of untreated control cells. The drug concentration which inhibits cell growth by 50% (IC_{50}) was determined from semi-logarithmic plots.

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