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Chloride anion transport and copper-mediated DNA cleavage by C-ring functionalized prodigiosenes†

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A new class of prodigiosenes with stability-enhancing functionalities appended to the C-ring were found to transport chloride anions through liposomal membranes, as well as to induce copper-mediated DNA cleavage.

Prodigiosin (**1**) is a red pigment produced by microorganisms such as *Streptomyces* and *Serratia* and was first isolated in 1929.[1] It has been studied extensively for its promising anticancer, antimicrobial and immunosuppressive activities.[2–4] Prodigiosin induces apoptosis in a variety of cell lines[5–8] and the DNA interaction and topoisomerase inhibition properties of prodigiosin have been studied.[9] Mechanisms have been proposed for the anticancer activity of prodigiosin: firstly, prodigiosin is an efficient H^+/Cl^- transporter[2,10] and a recent report confirmed that prodigiosin can transport chloride anions across lipid vesicles;[11] secondly, prodigiosin induces copper-mediated DNA cleavage.[12–18] The latter effect is proposed to be triggered by the formation of π-radical cations through oxidation of the electron-rich pyrrolylpyrromethene chromophore of **1** by the metal.[12] Prodigiosenes, derivatives of prodigiosin,[19] are being investigated as potential anticancer pharmaceuticals,[20–23] including published research involving three commercial ventures.[24–28]
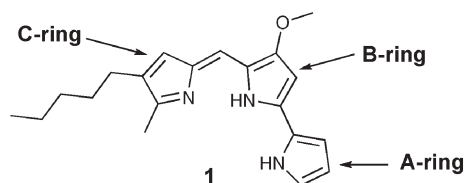
Synthetic prodigiosenes bearing pendant esters and β-carbonyl substituents conjugated to the C-ring were recently reported[29] by the Thompson group to generally retain the anticancer activity of prodigiosin in sixty human cell lines derived from nine cancer cell types, with the appended functionality not necessarily reducing the anticancer activity of the core skeleton. The prodigiosenes were synthesized by modification of D'Alessio's methodology,[30,31] using Knorr-type pyrroles to amend the C-ring. The β-carbonyl

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functionality enhances the stability of the prodigiosene core, and facilitates synthesis and isolation. The pendant ester moieties are intended as potential linking sites for the ultimate appendage of targeting moieties.[32] We herein report the efficiency of the new C-ring modified prodigiosenes **2–11** (Fig. 1) to transport chloride anions through liposomal membranes and to effect copper-mediated DNA cleavage. These studies are essential to determine whether these two suggested mechanisms of action for the anticancer activity of prodigiosin are feasible for prodigiosenes bearing β-carbonyl substituents and pendant esters.

Prodigiosin affects the acidification of cellular organelles and vesicles,[10,33] is a transmembrane chloride anion carrier, and has been used as a standard against which other transmembrane chloride transporters can be judged.[11] Amidopyrrolic mimics of prodigiosin exhibit efficient anion receptor and HCl membrane transport abilities, as well as anticancer activity.[25,26,28,34] To investigate prodigiosenes **2–11** for their ability to transport chloride ions across phospholipid membranes, chloride gradient assays using egg-yolk L-phosphatidylcholine (EYPC) liposomes at 25 °C were conducted. EYPC liposomes (100 nm diameter) containing 100 mM $NaNO_3$ (10 mM phosphate buffer pH 6.4) and 1.0 mM of the chloride-selective dye lucigenin[35] were prepared using standard protocols.[11] A solution of NaCl was then added to a suspension of EYPC liposomes in 100 mM $NaNO_3$–10 mM sodium phosphate buffer pH 6.4 so as to produce an extravesicular chloride concentration of 25 mM. After addition of the synthetic prodigiosene (at a ratio of 1 : 1000 prodigiosene : EYPC lipid) the fluorescence of the encapsulated lucigenin dye was monitored over time and fluorescence values were converted to chloride concentration.[11,35] The order of transport efficiency after 250 s was found
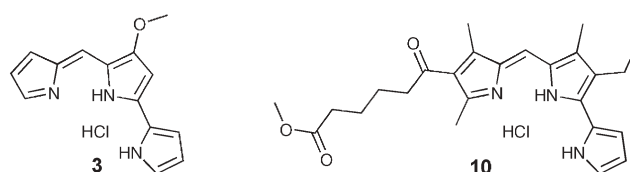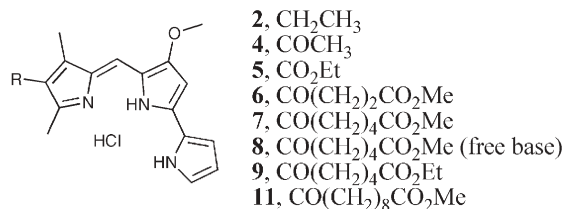

2, CH_2CH_3
4, $COCH_3$
5, CO_2Et
6, $CO(CH_2)_2CO_2Me$
7, $CO(CH_2)_4CO_2Me$
8, $CO(CH_2)_4CO_2Me$ (free base)
9, $CO(CH_2)_4CO_2Et$
11, $CO(CH_2)_8CO_2Me$

Fig. 1 Prodigiosenes **2–11**.

to be $2 \gg 11 \approx 5 > 10 \approx 6 \approx 3 > 9 > 4 > 7$ (Fig. S1), with all prodigiosenes exhibiting significant chloride ion transport ability at relatively low ligand : lipid concentrations (0.1 mol% relative to lipid). Typically, concentrations of synthetic chloride transporters of 1–2 mol% are needed to effect the same flux of transmembrane chloride transport shown by these prodigiosenes.[36]

To compare and contrast these results with a known compound, the assay was repeated using prodigiosin as a positive control[11] against prodigiosenes 2, 4 and 6 (all at a ratio of 0.1 mol% relative to EYPC lipid). Fig. 2 shows the results of the chloride ion transport studies for 2, 4 and 6, depicted as a plot of the chloride concentration inside the EYPC liposomes *versus* time. The synthetic prodigiosene 2 was as effective at chloride transport as was the natural product prodigiosin 1. The β-substituted prodigiosenes 4 and 6, though clearly less active than prodigiosin 1, still demonstrate significant transmembrane Cl^- transport activity under these assay conditions.[36]

With regard to correlations between prodigiosene structure and the ability to transport chloride ions across lipid membranes, prodigiosene 2 is the only derivative to retain the transport efficiency of 1. Structurally, both compounds are related in that they bear an alkyl chain in the same C-ring β-position. In contrast, introduction of other functionalities at the C-ring β-position (4–11), or the presence of no substituents at that and other positions (3), causes a modest decrease in the efficiency of chloride transport. This decrease in chloride transport rate may be due to a reduction in partitioning of the prodigiosenes into the lipid membrane, reduced diffusion across the bilayer membrane or because of a change in the affinity of the prodigiosenes for binding and release of chloride anion. This is the first report of liposomal chloride ion transport by a series of structurally related prodigiosenes. The most important finding of these transport experiments is that the prodigiosin core may be functionally altered without losing the ability to transport chloride ions across phospholipid membranes.

To investigate whether the copper-mediated DNA cleavage ability of prodigiosin[12,15–18] is maintained by prodigiosenes 2–11 agarose gel electrophoresis with supercoiled plasmid DNA (Fig. 3) was conducted in the presence of Cu(OAc)$_2$.[17] The agarose gels were used to calculate the EC_{50} values for each prodigiosene at

Fig. 3 A representative agarose gel showing the extent of DNA cleavage produced by an acetonitrile : water (1 : 1) solution of prodigiosene 8 : Cu(OAc)$_2$ (1 : 1) at 90 min after incubation at 37 °C. Reaction mixtures (20 μL total, 10 μL loaded in each gel) contained 450 ng of supercoiled DNA in 10 mM MOPS buffer, pH 7.4, and 100 mM NaCl. Lanes 1 and 2 are control experiments consisting of supercoiled DNA incubated with only 37 μM Cu^{2+} or only 37 μM prodigiosene 8, respectively; lanes 3–11 represent supercoiled DNA incubated with 0.5, 2, 5, 10, 15, 20, 25, 30 or 50 μM, respectively, prodigiosene 8 : Cu^{2+} (1 : 1).

30 min and 90 min, where EC_{50} represents the concentration of compound required to effect 50% DNA cleavage. After 30 min incubation, significant DNA cleavage was observed and the calculated EC_{50} values were 9–24 μM (Table 1) for all prodigiosenes, as well as for the parent prodigiosin (1). After a further 60 min incubation period, the range of EC_{50} values was reduced to 7–12 μM. As expected, neither Cu(OAc)$_2$ alone nor prodigiosene alone effected DNA cleavage (Fig. 3; lanes 1 and 2, respectively).

Clearly, functionality appended to the C-ring in 2–11 does not limit the ability of any of the prodigiosenes to cleave DNA, although cleavage was incomplete after 30 min and complete after 90 min incubation. The improvement in EC_{50} at 90 min for the five analogues 2–6 without a relatively long alkyl chain (⩾ four carbon atoms) implies that these compounds catalyze DNA cleavage more slowly than the other prodigiosenes. This is the first report of a time-dependent study of copper-mediated DNA cleavage by a series of structurally related prodigiosenes, and it indicates that the prodigiosin core may be functionally altered without significantly diminishing DNA cleavage.

In summary, nine synthetic derivatives of prodigiosin and prodigiosin itself, for control purposes, have been examined for their ability to effect liposomal transmembrane transport of chloride anions and DNA cleavage. For chloride ion transport, 2 was the only prodigiosene to maintain the efficiency of prodigiosin, presumably due to similar substitution patterns. For the other prodigiosenes, the transport efficiency was decreased with the introduction of carbonyl groups conjugated to the prodigiosin skeleton (prodigiosenes 4–11), as well as with prodigiosene 3 which
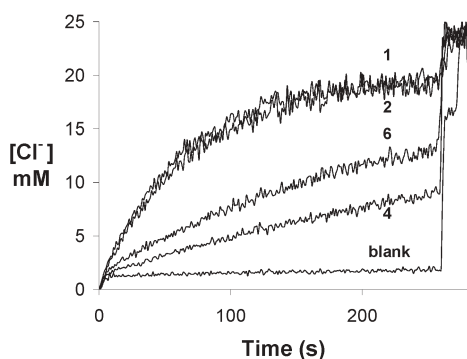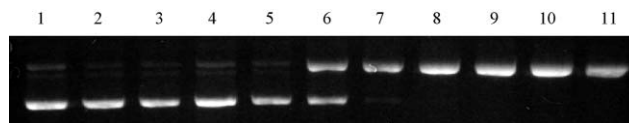

Fig. 2 Chloride transport across EYPC liposomes (25 °C) containing lucigenin in a 100 mM NaNO$_3$–10 mM sodium phosphate buffer (pH 6.4). Compounds 1, 2, 4 and 6 were added to give a 1 : 1000 ligand : lipid ratio. At $t = 0$ s, NaCl was added to give an external Cl^- concentration of 25 mM. Lucigenin fluorescence was converted to [Cl^-]. The traces shown are the average of 3 trials.

Table 1 EC_{50} values for prodigiosenes 1–11 after 30 and 90 min incubation at 37 °C with 1 : 1 prodigiosene : Cu^{2+}

Prodigiosene	EC_{50} (μM), 30 min	EC_{50} (μM), 90 min
1[a]	9.9 ± 1.4	9.2 ± 1.2
2	17.6 ± 9.2	8.2 ± 1.7
3	20.1 ± 0.2	9.6 ± 1.3
4	24.1 ± 6.3	12.4 ± 3.1
5	20.8 ± 9.1	10.3 ± 1.2
6	20.1 ± 0.2	9.6 ± 1.3
7	8.8 ± 2.1	8.8 ± 1.9
8[a]	10.8 ± 2.1	11.1 ± 0.2
9	9.7 ± 1.1	9.9 ± 0.8
10	8.4 ± 1.0	7.0 ± 0.9
11	10.0 ± 1.5	8.5 ± 0.3

[a] Free-base.

lacks an alkyl side-chain *cf.* prodigiosin. Most importantly, all of the prodigiosenes exhibit significant transmembrane transport of chloride at relatively low ligand : lipid concentrations. In the DNA cleavage assays the EC_{50} values for all prodigiosenes after 90 min incubation were spread over the range 7–12 μM, indicating that all the derivatives maintain the cleavage ability of prodigiosin (measured EC_{50} = 9.2 μM) and that new structural motifs appended to the C-ring do not significantly decrease this activity. However, analysis of EC_{50} values after 30 min indicated that the five analogues **2–6** lacking a long alkyl chain (*i.e.* ≥ four carbon atoms) in the C-ring cleave DNA more slowly than the other derivatives. Interestingly, EC_{50} values for these prodigiosenes do not parallel the chloride transport abilities, thus suggesting that chloride complexation and DNA cleavage are independent phenomena. These results will serve as cornerstones in the ongoing design of functionalized prodigiosenes with efficient and selective biological activity.

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