Ion Transport

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Synthesis, Anion-Binding Properties, and In Vitro Anticancer Activity of Prodigiosin Analogues**

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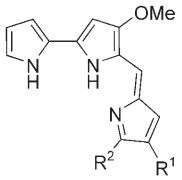
Prodigiosins, for example, **1** and **2**, are a family of naturally occurring tripyrrolic red pigments that were first isolated in

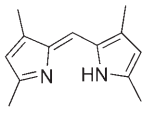


1: R^1 = H, R^2 = *n*-undecyl (prodigiosin 25-C)
2: R^1 = *n*-pentyl, R^2 = Me (prodigiosin)
3: R^1 = H, R^2 = Et

the 1930s from microorganisms including *Serratia* and *Streptomyces* and are characterized by a common pyrrolylpyrromethene skeleton.[1] These molecules, especially prodigiosin 25-C (**1**) but also synthetic analogues such as **3**,[2,3] have been studied extensively for their promising immunosuppressive[4] and anticancer activities.[5,6]

To date, two very different modes of action have been put forward to explain their anticancer activity. One suggestion, proposed by Manderville, Melvin, and co-workers,[7] and further supported by Fürstner and Grabowski,[8] is that prodigiosin mediates its anticancer effect through copper-mediated cleavage of double-stranded DNA. The second mechanistic suggestion, proposed by Ohkuma, Wasserman, and co-workers in 1998,[9] is that the biological activity of prodigiosin derives from its ability to effect concurrent transmembrane transport ("symport") of H^+ and Cl^- ions into cells.[9] Support for this mechanism came partly from the finding that the activity of prodigiosin as well as prodigiosin-based lysosomal acidification were dependent on the concentration of extracellular chloride ion and that chloride ion channels were not responsible for these effects.[9] However, to date, little in the way of direct chemical support for the mechanism has been provided.[10,11] Accordingly, we have undertaken a detailed study of the anion-binding and through-membrane transport properties of several prodigiosin analogues and corresponding dipyrromethene constituents. Herein, we report that it is the rate of transport, rather than the anion-binding ability, that correlates most closely with anticancer activity in vitro, as judged from cell proliferation assays involving A549 human lung and PC3 human prostate cancer cells.

To investigate the presumed anion binding and transport ability of prodigiosins, model compounds **3**–**7** were pre-



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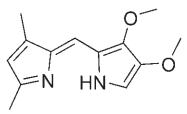

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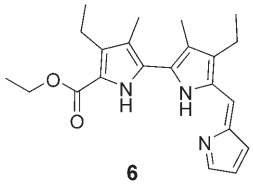

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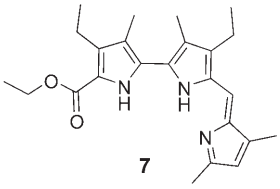

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pared.[12] These systems contain either the basic prodigiosin skeleton or the key constituent dipyrromethene motif and were thus thought to provide a "basis set" sufficient to allow some limited structure–activity correlations to be made.

The first indication that the protonated forms of prodigiosins and dipyrromethenes can bind chloride ions came from X-ray crystal structure analyses of salts **4**·HCl and **7**·HCl.[12] As seen in Figures 1 and 2, 1:1 complexes are formed in the solid state as the result of oriented hydrogen-bonding interactions and, presumably, electrostatic effects. In the case of salt **4**·HCl the complex is essentially flat, whereas

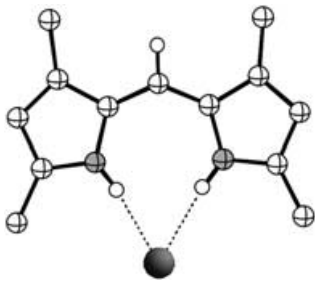

Figure 1. Crystal structure of **4**·HCl. This complex is essentially planar.

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

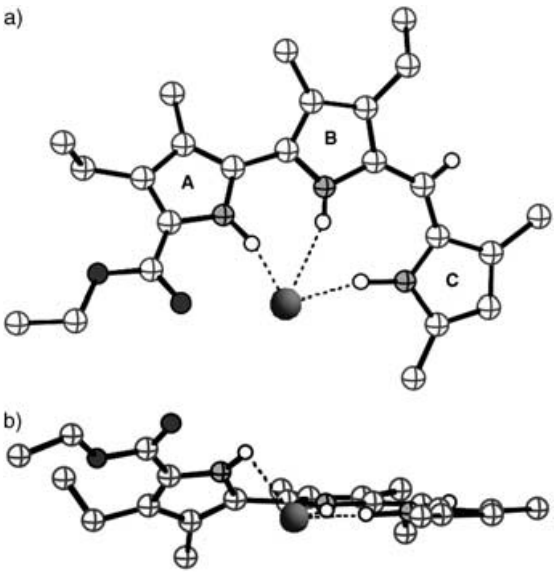

Figure 2. Two views of the crystal structure of the complex formed between monoprotonated prodigiosin **7** and chloride ion.

some deviation from planarity is seen in **7**·HCl. In **4**·HCl, the NH···Cl distances (2.25 Å) are roughly equal, while in the case of **7**·HCl they are found to be longer for ring A than rings B and C (NH···Cl = 2.72, 2.28, and 1.97 Å for rings A, B, and C, respectively).

Evidence in support of chloride ion binding in solution came from isothermal titration calorimetry (ITC) studies carried out in acetonitrile at 303 K using tetrabutylammonium chloride (TBACl) as the source of chloride ions. Whereas the free-base forms of prodigiosins **6** and **7** as well as various neutral bipyrroles were found to display affinities for chloride ion that were too low to be determined by ITC methods in CH_3CN, the corresponding monoprotonated HI salts of these prodigiosin species were found to exhibit rather substantial apparent affinities for chloride ion (see Table 1). The same proved true for the protonated dipyrromethene fragments **4** and **5**. As a rule, these latter compounds demonstrated slightly higher affinities for chloride ions than did the corresponding monoprotonated prodigiosins; a finding that reflects presumably the higher effective charge densities present in the smaller dipyrrolic species.

Table 1: Apparent association constants (K_a) for binding of chloride ion to the monoprotonated (HI) salts of dipyrromethenes and prodigiosin analogues **3**–**7** in CH_3CN, as determined by ITC analysis at 30 °C using TBACl as the anion source. Errors are estimated to be 10%.

	3	**4**	**5**	**6**	**7**
ΔH [kcal mol^{-1}]	−4.2	−3.35	−2.27	−1.59	−2.03
$T\Delta S$ [kcal mol^{-1}]	3.8	4.88	5.33	5.35	4.93
ΔG [kcal mol^{-1}]	−8.0	−8.23	−7.60	−6.94	−6.96
K_a [M^{-1}]	5.9×10^5	8.8×10^5	3.0×10^5	1.1×10^5	1.1×10^5

The efficiency of transport of chloride ions was determined by monitoring their efflux across a 200-nm POPC/POPS (POPC = 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS = 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine) liposome membrane as a function of time.[13] The vesicles were loaded with a solution of NaCl (500 mM) and then diluted to produce a 1 mM suspension in a solution of $NaNO_3$ (500 mM). The integrity of the liposome membranes was established according to reported procedures.[14] The efflux of chloride ion was monitored as a function of time using an Accumet glass-bodied chloride-selective electrode in conjunction with a Jenco multimeter. The results of the transport studies are summarized in Figure 3. Note that

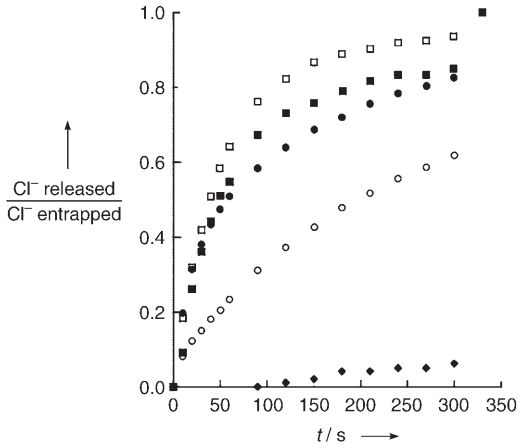

Figure 3. Time-dependent efflux of chloride ion from 200-nm vesicles loaded with a solution of NaCl (500 mM) and suspended in a solution of $NaNO_3$ (500 mM) and triethylsilane (5 mM), adjusted to pH 7.4. Prodigiosins are indicated by filled markers (**3** ●, **6** ■, **7** ◆); dipyrromethenes are indicated by open markers (**4** □, **5** ○).

tripyrrole **3**, the compound that bears the greatest structural similarity to the natural prodigiosins, demonstrated remarkably high transport efficiency. In fact, the rate of efflux of chloride ion was sufficiently rapid that studies were conducted using a 0.5 μM solution of the carrier. The remaining compounds were studied using 2 μM solutions. The order of transport efficiency, as inferred from initial rates, was found to be $\mathbf{3} \gg \mathbf{6} \approx \mathbf{4} > \mathbf{5} \gg \mathbf{7}$. Additionally, compounds **3**–**6** were all seen to prompt $\geqq 60\%$ release of chloride ions from the interior of the vesicles within 300 seconds.

To determine the mechanism of transport, the experiments were repeated with the pH of the external solution initially set at pH 5.5. The efflux of chloride ions was less efficient than when the external solutions were neutral or basic. This behavior is consistent with an H^+/Cl^- ion mechanism of transport, as it is expected that the driving force for proton egress would be reduced with a lower external pH value.

Experiments were also carried out in which 8-hydroxypyrene trisulfonate (HPTS), a pH-sensitive fluorescent dye,[15] was included within the liposomes and the external solution was basified using NaOH. The excitation maxima (λ_{max}) for HPTS are 450 (at pH 7–8) and 405 nm (pH < 6). Excitation ratio experiments were performed in which the sample was excited at both 405 and 450 nm while the emission

intensity was monitored at 511 nm. Following addition of the carrier, an increase in the peak at 450 nm and a corresponding decrease in the peak at 405 nm were observed. Upon lysis of the vesicles and subsequent contact with external hydroxide ion, these spectral changes became even more pronounced. In contrast, in the absence of chloride ion (but in the presence of carrier) no effects were seen. Such findings are also consistent with an H^+/Cl^- ion symport mechanism.

Finally, transport experiments were performed in which the external nitrate ion was replaced by sulfate ion. No significant changes in the transport rates or overall efficiency were observed under these conditions, as would be expected for anion efflux conducted through a H^+/Cl^- ion symport mechanism. However, if transport were to occur through an anion-exchange (antiport) mechanism, the transport rates would be expected to change as a function of the lipophilicity of the external anion.

Anticancer activity in vitro was assessed using a cell proliferation assay with A549 human lung cancer and PC3 human prostate cell lines.[16] The order of activity in vitro in both cell lines was found to be similar, namely, **3** > **6** > **4** > **5** > **7** (A549; see Figure 4) and **3** > **6** > **4** > **5** ≈ **7** (PC3; see

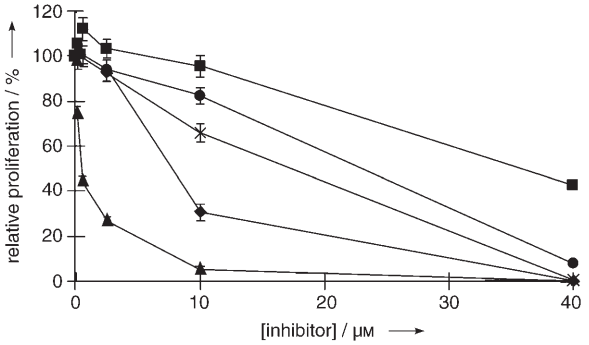

Figure 4. Antiproliferative activity of prodigiosin analogues and dipyrromethenes in A549 human lung cancer cells. Exponential phase cells were treated with compounds **3**–**7** at the indicated concentrations for 24 h (**3** ▲, **4** ×, **5** ●, **6** ◆, **7** ■). The number of viable cells was determined after 3 days using a standard tetrazolium salt reduction assay. Data are presented as the fraction of untreated cells (see Supporting Information for details).

Supporting Information). Compounds **3**, **4**, **5**, and **6** all exhibited significant cytotoxic activity, with 100% of cancer cells killed at a concentration of 40 μM in both cell lines. As observed earlier in the transport studies, compound **3** proved to be more efficient than the other compounds examined, displaying a notably greater antiproliferative effect.

Among the prodigiosin analogues examined in this study, the rates of through-liposome transport and in vitro activity were found to correlate well. A general sequence of efficiency of **3** > **6** ≥ **4** > **5** ≥ **7** is seen in both cases. In contrast, the values of the effective association constant (K_a) in Table 1 do not correlate well with the observed activity in vitro. For instance, compound **4**·H^+, which displays the highest overall K_a value, is far less active in cell culture than prodigiosin **3**. Such reduced activity could reflect an anion affinity that is too high to afford an optimal rate of release of chloride ion. Likewise, the low antiproliferative activity of **7** can be rationalized in terms of an anion affinity that is too low to make it a highly effective chloride ion carrier. The present findings thus provide support for a mechanism such as H^+/Cl^- ion symport that is dominated by kinetic rather than thermodynamic factors.

The present study does not address the issue of whether recruitment of copper and DNA modification plays a significant role in mediating the action of prodigiosin. However, the strong correlation between transport rates and anticancer activity in vitro, in conjunction with evidence for anion binding in the solid state and solution phase, lead us to suggest that the H^+/Cl^- ion symport mechanism proposed by Ohkuma, Wasserman, and co-workers[9] is chemically reasonable. If such a conclusion is correct, it leads to the suggestion that other chloride ion transport systems, including those that may have no direct structural resemblance to prodigiosins, may show interesting anticancer activity.

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[1] A. Fürstner, *Angew. Chem.* **2003**, *115*, 3706 – 3728; *Angew. Chem. Int. Ed.* **2003**, *42*, 3582 – 3603.
[2] R. D'Alessio, A. Bargiotti, O. Carlini, F. Colotta, M. Ferrari, P. Gnocchi, A. Isetta, N. Mongelli, P. Motta, A. Rossi, M. Rossi, M. Tibolla, E. Vanotti, *J. Med. Chem.* **2000**, *43*, 2557 – 2565.
[3] M. S. Melvin, J. T. Tomlinson, G. Park, C. S. Day, G. R. Saluta, G. L. Kucera, R. A. Manderville, *Chem. Res. Toxicol.* **2002**, *15*, 734 – 741.
[4] S. B. Han, H. M. Kim, Y. H. Kim, C. W. Lee, E.-S. Jang, K. H. Son, S. U. Kim, Y. K. Kim, *Int. J. Immunopharmacol.* **1998**, *20*, 1 – 13.
[5] D. Yamamoto, Y. Kiyozuka, Y. Uemura, C. Yamamoto, H. Takemoto, H. Hirata, K. Tanaka, K. Hioki, A. Tsubura, *J. Cancer Res. Clin. Oncol.* **2000**, *126*, 191 – 197.
[6] B. Montaner, R. Perez-Toma, *Life Sciences* **2001**, *68*, 2025 – 2036.
[7] a) M. S. Melvin, M. W. Calcutt, R. E. Noftle, R. A. Manderville, *Chem. Res. Toxicol.* **2002**, *15*, 742 – 748; b) G. Park, J. T. Tomlinson, M. S. Melvin, M. W. Wright, C. S. Day, R. A. Manderville, *Org. Lett.* **2003**, *5*, 113 – 116.
[8] A. Fürstner, E. J. Grabowski, *ChemBioChem* **2001**, *2*, 706 – 709.
[9] a) T. Sato, H. Konno, Y. Tanaka, T. Kataoka, K. Nagai, H. H. Wasserman, S. Ohkuma, *J. Biol. Chem.* **1998**, *273*, 21455 – 21462; b) S. Ohkuma, T. Sato, M. Okamoto, H. Matsuya, K. Arai, T. Kataoka, K. Nagai, H. H. Wasserman, *Biochem. J.* **1998**, *334*, 731 – 741.
[10] In previous work, the HCl complexes of extended "vinylogous" prodigiosin analogues,[17] a cyclic prodigiosin derivative,[18] and a phenyl-substituted dipyrromethene derivative[3] were reported. While single-point binding of chloride ion is seen in the case of the latter system, the first two structures revealed binding modes that are analogous to those seen in the case of **4**·HCl and **7**·HCl, respectively.
[11] During the course of preparing this manuscript, we became aware of a parallel study of H^+/Cl^- ion symport using a different set of prodigiosin analogues, namely a set of wholly synthetic pyrrole amides. See: P. A. Gale, M. E. Light, B. McNally, K. Navakhun, K. E. Sliwinski, B. D. Smith, *Chem. Commun.* **2005**,

3773 – 3775. We thank the authors for making their manuscript available to us prior to publication.
[12] Experimental details and characterization data are included in the Supporting Information. CCDC 271587 and 271586 (**4**·HCl and **7**·HCl, respectively) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.
[13] A. Koulov, T. N. Lambert, R. Shukla, M. Jain, M. Boon, B. D. Smith, H. Li, D. N. Sheppard, J.-B. Joos, J. P. Clare, A. P. Davis, *Angew. Chem.* **2003**, *115*, 5081 – 5083; *Angew. Chem. Int. Ed.* **2003**, *42*, 4931 – 4933; .
[14] a) F. Nicol, S. Nir, F. C. Szoka, Jr., *Biophys. J.* **2000**, *78*, 818 – 829; b) J. P. Koniarek, J. L. Thomas, M. Vazquez, *Adv. Space Res.* **2004**, *34*, 1373 – 1377; c) M. Castaing, A. Loiseau, L. Djoudi, *Eur. J. Pharm. Sci.* **2003**, *18*, 81 – 88; d) A. G Krishna, S. T. Menon, T. J. Terry, T. P. Sakmar, *Biochemistry* **2002**, *41*, 8298 – 8309.
[15] K. Kano, J. H. Fendler, *Biochim. Biophys. Acta* **1978**, *509*, 289 – 299.
[16] T. Mosmann, *J. Immunol. Methods* **1983**, *65*, 55 – 63.
[17] A. Treibs, M. Strell, I. Strell, D. Grimn, *Justus Liebigs Ann. Chem.* **1978**, 289 – 305.
[18] A. Fürstner, J. Grabowski, C. W. Lehmann, *J. Org. Chem.* **1999**, *64*, 8275 – 8280.

