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Perenosins: a new class of anion transporter with anti-cancer activity†

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A new class of anion transporter named 'perenosins' consisting of a pyrrole linked through an imine to either an indole, benzimidazole or indazole is reported. The indole containing members of the perenosin family function as effective transmembrane $\text{Cl}^-/\text{NO}_3^-$ antiporters and HCl cotransporters in a manner similar to the prodigiosenes. The compounds reduce the viability of MDA-MB-231 and MCF-7.

Introduction

Prodigiosin is naturally occurring tripyrrolic compound,¹ produced by a group of microorganisms including *Serratia marcescens* (Fig. 1).² Although the compound was first isolated in pure form in 1929,³ it was not until 1977 that Fullan and co-workers demonstrated that prodigiosin has anti-tumour activity. Since that time the anti-cancer properties of many different natural and synthetic prodigiosenes have been explored.^{4,5} Prodigiosin has been shown to passively transport HCl across lipid bilayer membranes and it is proposed that the anti-cancer properties of this class of compounds may be linked to this process.^{6,7} Many prodigiosenes have also shown potent antimicrobial,⁸ antimalarial⁹ and immunosuppressive activity.¹⁰ Unfortunately, the high toxicity of prodigiosin and its analogues prevents their use in the clinic.¹¹ Closely related

compounds including the tambjamins^{12,13} and obatoclax,¹⁴ having similar structures, have been shown to exhibit similar biological properties (Fig. 1). Obatoclax mesylate GX15-070, an indole-based prodigiosin analogue, is currently in clinical trials, being evaluated in solid tumors and hematological neoplasms.

Pyrrole and indole groups are found in many synthetic anion receptor systems. Examples that have been employed in lipid bilayer anion transport include amidopyrrole functionalized with a basic methylimidazole group that was shown to co-transport HCl,¹⁵ calixpyrrole-based transporters¹⁶ including strapped systems that trigger apoptosis in cells due to influx of NaCl ,¹⁷ and indole functionalized thioureas.¹⁸ This latter class of compound have also been used as carboxylate transporters.¹⁹

In this paper we report the synthesis of a new class of anion transporter with structures inspired by prodigiosin. Known as 'perenosins',²⁰ these compounds contain a pyrrole hydrogen bond donor linked through an imine to an indole, benzimidazole or indazole. Compounds with a range of lipophilicities have been prepared and their anion complexation and transport properties studied.

Results and discussion

Synthesis and characterization

Perenosins **1a–e**, **2** and **3** (Fig. 2) were prepared using a condensation reaction (EtOH , MgSO_4 , room temperature, 24 h) of 3,5-dimethylpyrrole-2-carboxaldehyde with a reduced 7-nitroindole, 7-nitrobenzimidazole or 7-nitroindazole (H_2 , Pd/C 10%, EtOH , room temperature, 5 h). The non-commercial 7-nitroindoles (except for $\text{R} = \text{MeO}$) were readily obtained from the respective 2-nitroaniline starting material through an iodination – Sonagashira reaction – base assisted cyclization pathway. For 5-methoxy-7-nitroindole an alternative Fisher indole synthesis – decarboxylation route was followed. Further details are provided in the ESI.†

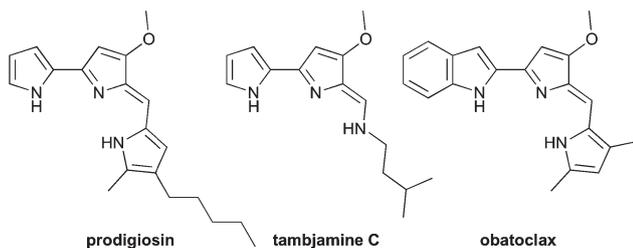


Fig. 1 Prodigiosin, tambjamine C and obatoclax.

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† Electronic supplementary information (ESI) available: Syntheses details, ¹H NMR and ¹³C NMR spectra, X-ray crystallographic data, vesicle transport assay details, Hill plots, hydrolysis data, *in vitro* assays details, and other supporting figures. Data underlying this publication are available see: DOI: 10.5258/SOTON/386610. CCDC 1441636. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/C6OB00002A



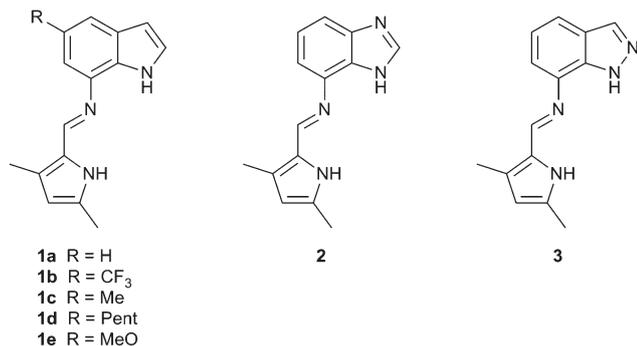


Fig. 2 The structures of the perenosins reported in this paper.

Compounds **1a–e**, **2** and **3** all obey ‘Lipinski’s rule of 5’ (except the non-protonated form of compound **1d** which has a $\log P$ slightly over 5).²³ The $\log P$ of **1a–e**, **2**, **3** were calculated with VCClLabs (Table 1).²² Although initially hypothesised by J. T. Davis *et al.*, no direct correlation between the basicity of prodigiosenes and their anti-cancer properties was found.²⁴ pK_a is, however, an indication at which pH the compounds are protonated and therefore at what point an increased affinity for anions is to be expected. The apparent pK_a values for **1a–e** and **2** were determined *via* a spectrophotometric method, previously described by Manderville (Fig. 3 and Table 1).²⁵ In solution, protonated perenosins are dark yellow to orange with absorbance maxima above 380 nm. As free-base the compounds are mostly lightly yellow and absorb at a lower wavelength. Gradual variation of pH allows monitoring of the change in ionization state and determination of the pK_a values from plots of $\log(\text{Abs})$ versus pH (see ESI†).

X-ray crystallographic analysis

A single crystal of **1a** with HCl suitable for X-ray analysis was obtained *via* slow evaporation of a CDCl₃ solution of **1a** in the presence of a small excess of HCl. The structure (Fig. 4) reveals that the protonated perenosin **1a** forms three hydrogen bonds to chloride to form a 1 : 1 complex (N–Cl distances 3.169(4)–

Table 1 Experimentally determined EC_{50/270}, Hill coefficient (*n*), and pK_a and calculated $\log P$ ($\log P$) (free-base and protonated form) for perenosins **1a–e**, **2** and **3**

Transporter	pK_a	EC _{50/270} ^b (mol%)	<i>n</i>	$\log P$ (error) ^c	$\log P$ protonated (error) ^c
1a	6.84	0.0773	1.11	2.98 (±0.60)	2.05 (±1.20)
1b	5.38	1.1922	1.52	3.84 (±0.61)	3.37 (±0.59)
1c	6.81	0.0301	1.00	3.36 (±0.66)	2.41 (±1.17)
1d	6.65	0.0299	1.00	5.16 (±0.98)	4.02 (±1.46)
1e	7.11	0.1859	1.33	2.85 (±0.74)	2.00 (±1.16)
2	7.18	6.4084	1.93	2.32 (±0.44)	1.58 (±1.38)
3	n.d. ^d	5.4305	2.21	2.44 (±0.50)	1.48 (±1.26)
Prodigiosin	7.16 ^a	0.0002	1.00	4.12 (±0.78)	3.28 (±1.15)

^a Literature value.²¹ ^b Cl[−]/NO₃[−] assay using POPC/chol (7 : 3) at pH 7.2.
^c Values calculated with VCClLabs.²² ^d not determined.

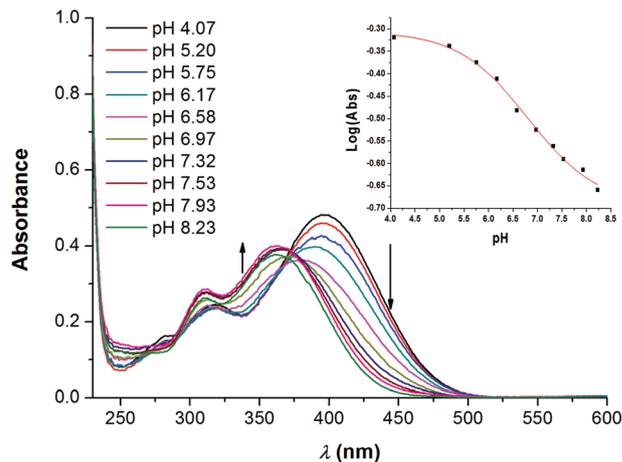


Fig. 3 UV-Absorbance spectra for **1c** (added as DMSO solution) as a function of pH in phosphate buffer at 20 °C (0.1 M NaCl). Due to solubility issues minor spectral deviations were observed at some pH values.

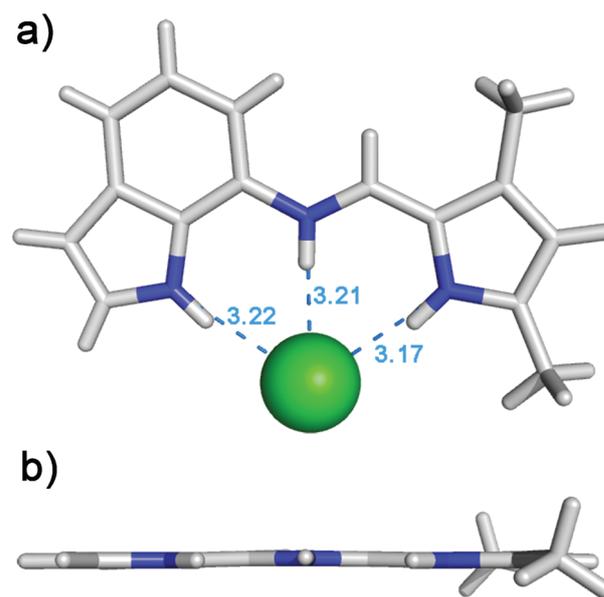


Fig. 4 X-ray crystal structure of [**1a** + HCl]; (a) indicating the distance (given in Å) between donor and acceptor; (b) side view (Cl[−] omitted for clarity).

3.223(3) Å, N–H...Cl angles 164.3–175.5°). The compound adopts a planar conformation allowing for conjugation throughout the molecule and a more rigid structure (Fig. 4b).

¹H NMR titration studies

To assess the affinity for the biologically important chloride and bicarbonate anions, ¹H NMR titration studies were performed to obtain association constants (1 × 10^{−5} M DMSO-*d*₆/0.5% H₂O, 298 K). The apparent association constants were calculated using WinEqNMR 2²⁶ by fitting the titration data to a 1 : 1 binding model, as was found from Job plot analysis²⁷ supported by the single-crystal X-ray analysis (Table 2). Upon



Table 2 Association constants K_a (M^{-1}) for 1:1 complexation between **1a–e**, **2**, **3** (1×10^{-5} M, 298 K) and anionic guests in DMSO- d_6 /0.5% H_2O^D

Receptor	Cl^- (M^{-1}) (1 equiv. HPF_6^-) ^b	Cl^- (M^{-1})	HCO_3^- (M^{-1})
1a	1340	<5 ^c	8.98
1b	4030	6.98	Deprot.
1c	1670	<5 ^c	9.50
1d	1650	<5 ^c	11.5
1e	2320	<5 ^c	14.4
2	318		Deprot.
3	433	<5 ^c	Deprot.

^a Calculated using WinEqNMR2.²⁶ Maximum error estimated to be $\pm 15\%$. ^b The exchange between PF_6^- and Cl^- is observed. ^c Only minor spectral changes were observed under these conditions.

stepwise addition of chloride to the protonated host [**1a** + HPF_6^-] the pyrrole N–H (11.91 to 13.28 ppm), iminium N–H (11.82 to 13.00 ppm) and indole N–H (11.37 to 11.57 ppm) proton resonances shifted downfield. In addition, a substantial downfield shift for the indole proton in the 6-position (7.33 to 7.59 ppm) and a modest downfield shift for the imine C–H proton (8.64 to 8.71 ppm) was observed. For the remaining protons no significant changes in chemical shift were observed. Addition of $TEAHCO_3$ or $TBAH_2PO_4$ to the protonated [**1a** + HPF_6^-] resulted in deprotonation of the receptor, whereas upon addition of $TBANO_3$ no notable changes in chemical shift were observed. Similar results have previously been observed with prodigiosin.²⁸ Receptors **1b–e** responded in a similar manner to the addition of chloride and were shown to possess a similar affinity for chloride. For compounds **2** and **3**, having benzimidazole and indazole functionalities instead of the indole group, respectively, the association constant with chloride was 10 times lower presumably due to the different resonance forms present (see ESI[†]).

Addition of $TBACl$ to **1a–e**, **2**, **3** (1×10^{-5} M DMSO- d_6 /0.5% H_2O , 298 K) only perturbed the proton resonances minimally not allowing for an association constant to be calculated in this competitive solvent mixture (Table 2). Addition of $TEAHCO_3$ to the free-base form of the receptors revealed the presence of a very weak interaction, presumably due to the more basic nature of the anion and the potential binding of the anion's proton to the imine functionality.

Transmembrane chloride transport studies

The anti-cancer properties of the prodigiosenes have been linked to their ability to transport passively chloride or H^+/Cl^- across vesicle and cell membranes.^{6,7} Consequently, the ability of the perenosins to facilitate chloride and proton transport across lipid bilayers was assessed using a combination of ion selective electrode (ISE) and fluorescence assays. To quantify the chloride efflux rate Hill plots were determined for 200 nm POPC:cholesterol liposomes (Table 1; see ESI[†]). Typically, unilamellar vesicles were prepared from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and cholesterol (7:3 ratio), containing an intravesicular sodium chloride solution (489 mM with 5 mM phosphate buffer at pH 7.2), were

suspended in an isotonic sodium nitrate solution (489 mM with 5 mM phosphate buffer at pH 7.2). Perenosins **1a–e**, **2**, **3** were added as a DMSO solutions and the resulting chloride efflux was monitored by a chloride selective electrode. At the end of the experiment detergent (octaethylene glycol monododecyl ether) was added to lyse the liposomes and calibrate the electrode to 100% chloride release.

This assay and others described below are evidence that the perenosins are mediating chloride/nitrate antiport in this case. Through the addition of transporter **1a** in various concentrations, a Hill plot²⁹ was derived giving $EC_{50\ 270\ s}$ of 0.0773 mol% (carrier to lipid). The more electron-deficient analogue **1b** ($R = CF_3$), having a higher affinity for chloride but a lower pK_a , proved to be a less efficient chloride transporter with $EC_{50\ 270\ s}$ of 1.1922 mol% (Fig. 5). The introduction of a methyl or pentyl substituent in the 5-position of the indole moiety provided a decrease of the $EC_{50\ 270\ s}$ value ($EC_{50\ 270\ s}$ 0.0301 and 0.0299 mol%, respectively). Presumably the increase in $\log P$ with respect to **1a** resulted in improved chloride efflux. A higher pK_a via the use of methoxy-derivative **1e** which should be protonated more easily and is therefore expected to transport more efficiently, resulted in a less effective transporter than **1a–d** ($EC_{50\ 270\ s}$ 0.1859 mol%) most probably attributed to the receptor's lower $\log P$ value (Table 1). The benzimidazole and indazole derivatives were shown to be poor lipid bilayer chloride transporters. Perenosin **1d** with the lowest $EC_{50\ 270\ s}$ was found to be two orders of magnitude slower than prodigiosin ($EC_{50\ 270\ s}$ 0.0299 and 0.0002, respectively).

To investigate the effect of the pH on the transport activity of perenosins **1a–e** and **2**, chloride/nitrate antiport was followed at different pH values (pH 4.0, 6.2, 7.2 and 8.2; Fig. 6). Upon decreasing the pH from 7.2 to 6.2, an increase in transport is observed corresponding to the increased amount of

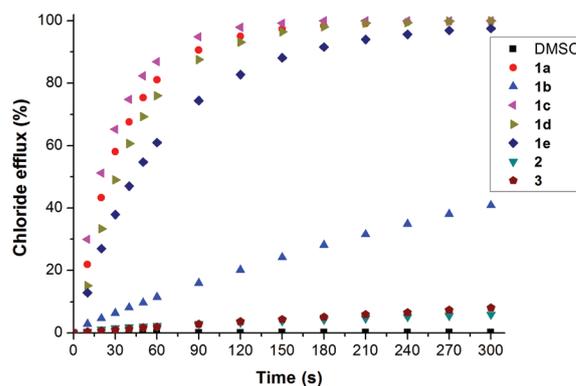


Fig. 5 Chloride efflux promoted by a DMSO solution of compound **1a–e**, **2**, **3** (1 mol% carrier to lipid) from unilamellar POPC:cholesterol vesicles loaded with 489 mM NaCl buffered to pH 7.2 with 5 mM sodium phosphate salts. The vesicles were dispersed in 489 mM $NaNO_3$ buffered to pH 7.2 with 5 mM sodium phosphate salts. At the end of the experiments, detergent was added to lyse the vesicles and calibrate the ISE to 100% chloride efflux. Each point represents the average of three trials. DMSO was used as a control.



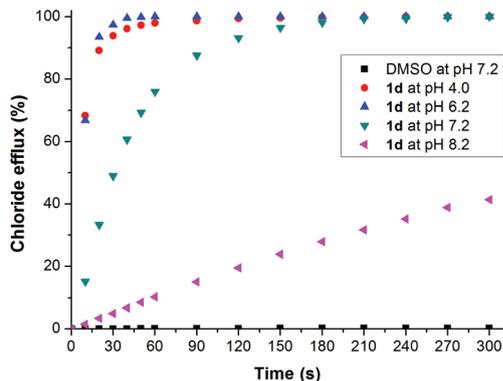


Fig. 6 Anion exchange assay promoted by a DMSO solution of compound **1d** (1 mol% carrier to lipid) from unilamellar POPC : cholesterol vesicles loaded with 489 mM NaCl buffered to a given pH with 5 mM sodium phosphate salts (pH 7.2 and 8.2), piperazine (pH 6.2) or citric acid (pH 4.0). The vesicles were dispersed in 489 mM NaNO₃ buffered to a given pH with 5 mM sodium phosphate salts (pH 7.2 and 8.2), piperazine (pH 6.2) or citric acid (pH 4.0). At the end of the experiments, detergent was added to lyse the vesicles and calibrate the ISE to 100% chloride efflux. Each point represents the average of three trials. DMSO was used as a control.

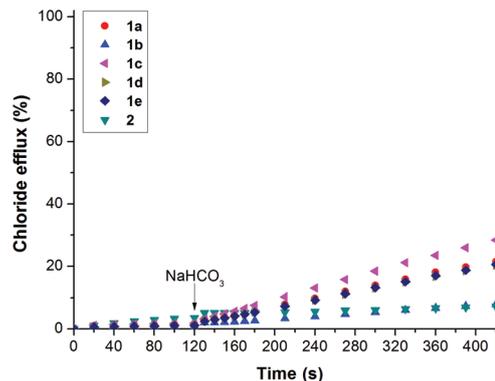


Fig. 7 Change of extravesicular chloride concentration over time for **1a–e**, **2** (1 mol% carrier to lipid) using unilamellar POPC : cholesterol vesicles loaded with 489 mM NaCl buffered to pH 7.2 with 20 mM sodium phosphate salts. The vesicles were dispersed in 167 mM Na₂SO₄ buffered to pH 7.2 with 20 mM sodium phosphate salts. At $t = 120$ s, a solution of NaHCO₃ spike was introduced such that the external NaHCO₃ concentration was 40 mM. At the end of the experiments, detergent was added to lyse the vesicles and calibrate the ISE to 100% chloride efflux. Each point represents the average of three trials.

receptor molecules being protonated. At pH 8.2 there is a significant drop in transport activity as presumably a significant proportion of the transporters are not protonated. This is evidence that only the protonated form of this perenosin is capable of transporting anions.

To further explore the transport mechanism operating in this system, a variety of ISE and fluorescence vesicle assays were performed altering the bilayer and the intra- or extravesicular solution composition. To probe whether metal ion–anion symport occurs POPC vesicles were loaded with different group 1 metal (Na⁺, K⁺, Cs⁺) chloride salts (see ESI†). The metal was found to have no effect on the rate of chloride efflux from the vesicles upon addition of compound **1a**, evidence in support of a transport mechanism not involving metal cations.

The sulfate ion is highly hydrophilic and is more challenging to transport across the lipid bilayer than nitrate.³⁰ Upon addition of perenosin to vesicles loaded with sodium chloride suspended in a sodium sulfate solution, no chloride efflux was observed (see ESI†). Upon addition of bicarbonate to the extravesicular solution, a chloride/bicarbonate antiport mechanism may be initiated. After the bicarbonate pulse at $t = 120$ s, a modest increase in extravesicular chloride concentration was noted (Fig. 7). The compounds proved to be quite poor bicarbonate transporters presumably due to deprotonation of the protonated perenosin. This is evidence in support of the protonated form of the receptor being the species that is capable of transporting anions across the bilayer.

In the sulfate assays no anion transport was observed, however due to the very small intravesicular volume, HCl co-transport along a pH gradient using ion-selective electrode assays is very hard to quantify. The possible presence of HCl co-transport was studied by fluorescence using a pH gradient assay. Vesicles containing sodium chloride (489 mM) and

1 mM 8-hydroxy-1,3,6-pyrenetrisulfonate (HPTS), a pH sensitive fluorescent dye were prepared.³¹ The vesicles were suspended in a solution of sodium sulfate (167 mM) and the HPTS fluorescence measured upon addition of a DMSO solution of compounds **1a–e**, **2** (Fig. 8). An increase in pH was observed, corresponding to the deacidification of the vesicles *via* a H⁺/Cl[−] co-transport mechanism (with Cl[−]/OH[−] antiport being ruled out due to the decrease in transport observed at higher pH and with basic anions such as bicarbonate).

Prodigiosenes have been shown to transport HCl across the lipid bilayer *via* a mobile carrier mechanism.¹ The Hill coefficients found for all indole-based perenosins and prodigiosin have a value of approximately 1 evidence in support of the hypothesis that the transport of a chloride ion can be

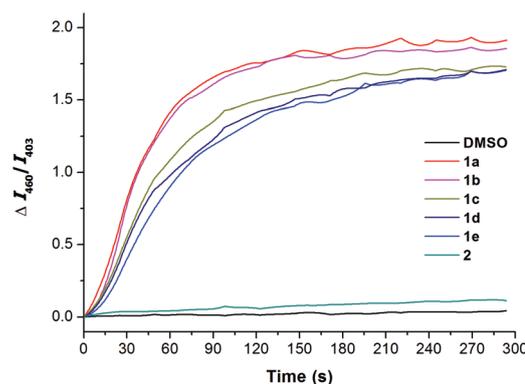


Fig. 8 Fluorescence assay ($\lambda_{\text{ex}} = 403$ and 460 nm, $\lambda_{\text{em}} = 510$ nm) for **1a–e**, **2** (1 mol% carrier to lipid) using unilamellar POPC : cholesterol vesicles loaded with 489 mM NaCl buffered to pH 7.2 with 20 mM sodium phosphate salts and 1 mM HPTS. The vesicles were dispersed in 167 mM Na₂SO₄ buffered to pH 7.2 with 20 mM sodium phosphate salts. Each point represents the average of three trials.



performed by a single carrier molecule.³² The non-indole perenosins **2** and **3** have a Hill coefficient of 1.93 and 2.21, respectively, evidence in support of cooperative mechanism involving two carrier molecules transporting one chloride ion.

Evidence for a carrier mechanism was derived from U-tube experiments.³³ Transporters **1a**, **1c–e**, **2** as a solution in chloroform (1 mM) were kept between two aqueous phases as a membrane model mimicking a vesicle assay (see ESI†). The source aqueous phase was loaded with sodium chloride (489 mM buffered to pH 7.2 with 5 mM sodium phosphate salts) and the receiving aqueous phase was loaded with sodium nitrate (489 mM buffered to pH 7.2 with 5 mM sodium phosphate salts). The large separation between the two aqueous phases rules out the possibility of transport *via* channel formation. Chloride transport was monitored using an ISE and showed that all the tested perenosins yielded an increase in chloride concentration in the receiving phase over time (5 days). These results support the hypothesis of a mobile carrier mechanism being the most likely mode of transport in this case.

Transport studies with the prodigiosenes show that the pK_a of the transporter correlates well with the $EC_{50,270s}$,²⁴ however no clear correlation could be found between the pK_a of perenosins and their $EC_{50,270s}$. However, compounds with a pK_a value higher than 6.65 and a $\log P$ between than 2.85 and 5.16, (supported by the $\log P$ range stipulated by Quesada *et al.*,^{13b}) appear to exhibit the best chloride transport properties.

Taking all the transport studies together the results show that the indole perenosins behave similarly to prodigiosin namely functioning as both a HCl cotransporter and a Cl^-/NO_3^- antiporter⁶ and forming a 1 : 1 complex with the anion.

Hydrolysis studies

The rate of hydrolysis is an important factor within the set of pharmacokinetic properties, the collective of bioavailability and processes of absorption, distribution, metabolism and elimination. As a model for all perenosins, the hydrolysis rate of **1a** in phosphate buffer (0.1 M NaCl) was determined following the perturbation of the UV/Vis spectroscopic data over time (see ESI†). At pH 7.2 the half-life time of **1a** was calculated, following first-order kinetics, to be 10.8 h.³⁴ Lowering the pH to 4.0 shortened the half-life time of **1a** to 6.6 h, consistent with the presence of the imine linker. Entrapment of **1a** inside a vesicular lipid bilayer at pH 7.2 extended the life span of perenosin **1a** six fold (half-life 60.2 h). Vesicles that fuse with the cancer cell membrane, potentially decorated with cancer cell selective receptors and fluorophores, therefore offer a potential route to administer future anti-cancer agents based upon the perenosin scaffold. An additional benefit would be the reduced toxicity for highly active compounds when administered whilst embedded inside liposomes.³⁵

Cell-based analysis

We performed preliminary studies to assess the effect of perenosins on the viability of cancerous and non-cancerous model cell lines. Compounds **1a–e**, **2** were assessed for their effect on

Table 3 IC_{50} (μM) values for **1a–e**, **2** on MDA-MB-231, MCF-7 and MCF-10A cells

Compound	MDA-MB-231 (μM)	MCF-7 (μM)	MCF-10A (μM)
1a	9.07 \pm 1.30	6.02 \pm 1.27	15.43 \pm 5.75
1b	3.67 \pm 0.05	4.13 \pm 0.22	12.93 \pm 3.23
1c	5.10 \pm 1.08	4.38 \pm 0.30	11.92 \pm 3.37
1d	4.39 \pm 0.80	3.84 \pm 0.12	22.37 \pm 7.74
1e	9.92 \pm 0.80	5.61 \pm 1.48	18.28 \pm 6.69
2	28.78 \pm 2.33	20.22 \pm 7.35	51.20 \pm 15.31

the viability of breast carcinoma MDA-MB-231 (invasive) and MCF-7 (non-invasive) model cell lines, as well as MCF-10A normal mammary model cells (Table 3). The cell lines were treated with increasing doses of perenosins for 24 h and the effect on the degree of cell viability was determined by MTT assays giving a dose–response curve (see ESI Fig. S85–S90†) that was used to determine the IC_{50} values for each compound in each cell line.

All indole-based perenosins **1a–e** were cytotoxic to the two malignant cell lines at low μM , with **1b**, **1c** and **1d** being most potent. All the molecules tested here were less potent in the normal MCF-10A cells. Interestingly, **1d** showed the largest selectivity (~ 5.5 fold) for the cancerous cell lines tested here. The benzimidazole derivative **2** was found to be substantially less active (IC_{50} 24.32 μM) than the other molecules. The most active compounds in cells (**1b** and **1d**) were also the most lipophilic in the series ($\log P$ 3.84 and 5.06, respectively). The reduced cytotoxicity observed in the normal MCF-10A cells suggests a potential mechanism for selective targeting of cancer cells with more potent derivatives of these molecules.

Conclusions

The perenosins represent a new class of highly effective anion transporters based upon the structure of prodigiosin. It has been demonstrated that indole-based perenosins are highly efficient chloride transporters and function as a mobile-carrier by an antiport and H^+/Cl^- symport mechanism of anion transport. The most lipophilic derivatives affect the viability of two breast cancer cell lines with ~ 5.5 -fold selectivity over normal breast cells. These compounds therefore represent excellent lead structures for further exploration of the potentially selective anti-cancer activities of this new class of molecules.

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