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Anion Transporters and Biological Systems

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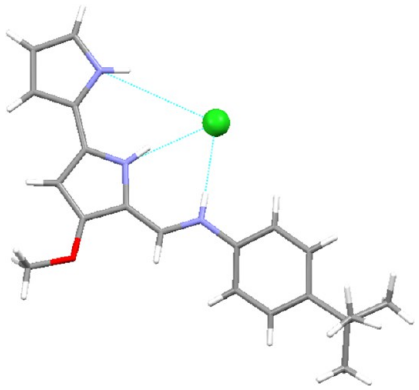
CONSPECTUS

In this Account, we discuss the development of new lipid bilayer anion transporters based on the structure of anionophoric natural products (the prodigiosins) and purely synthetic supramolecular systems. We have studied the interaction of these compounds with human cancer cell lines, and, in general, the most active anion transporter compounds possess the greatest anti-cancer properties.



Initially, we describe the anion transport properties of synthetic molecules that are based on the structure of the family of natural products known as the prodiginines. Obatoclax, for example, is a prodiginine derivative with an indole ring that is currently in clinical trials for use as an anti-cancer drug. The anion transport properties of the compounds were correlated with their toxicity toward small cell human lung cancer GLC4 cells. We studied related compounds with enamine moieties, tambjamines, that serve as active transporters. These molecules and others in this series could depolarize acidic compartments within GLC4 cells and trigger apoptosis. In a study of the variation of lipophilicity of a series of these compounds, we observed that, as log *P* increases, the anion transport efficiency reaches a peak and then decreases.

In addition, we discuss the anion transport properties of series of synthetic supramolecular anion receptor species. We synthesized trisureas and thioureas based on the tren backbone, and found that the thiourea compounds effectively transport anions. Fluorination of the pendant phenyl groups in this series of compounds greatly enhances the transport properties. Similar to our earlier results, the most active anion transporters reduced the viability of human cancer cell lines by depolarizing acidic compartments in GLC4 cells and triggering apoptosis.

In an attempt to produce simpler transporters that obey Lipinski's Rule of Five, we synthesized simpler systems containing a single urea or thiourea group. Once again the thiourea systems, and in particular a thiourea with a pendant indole group, transported anions efficiently. A series of related compounds containing a pendant trifluoromethyl group showed enhanced transport and significant anticancer properties.

Researchers still need to determine of the exact mechanism of how these compounds depolarize acidic organelles within cancer cells. However, this work shows that these transporters based upon both natural products and purely synthetic supramolecular systems transport anions, depolarize acidic compartments within cancer cells and trigger apoptosis.

1. Introduction

The development of synthetic receptors for anions is a maturing field with many examples of selective receptors that employ hydrogen bonds, electrostatic interactions, halogen bonds, anion−π interactions, and Lewis acid−anion interactions appearing in the literature.[1] By contrast, our understanding of how to design effective lipid bilayer transporters for anions is at an earlier stage.[2−4] The dysregulation of anion transport across lipid bilayer membranes can lead to a number of diseases including cystic fibrosis, the

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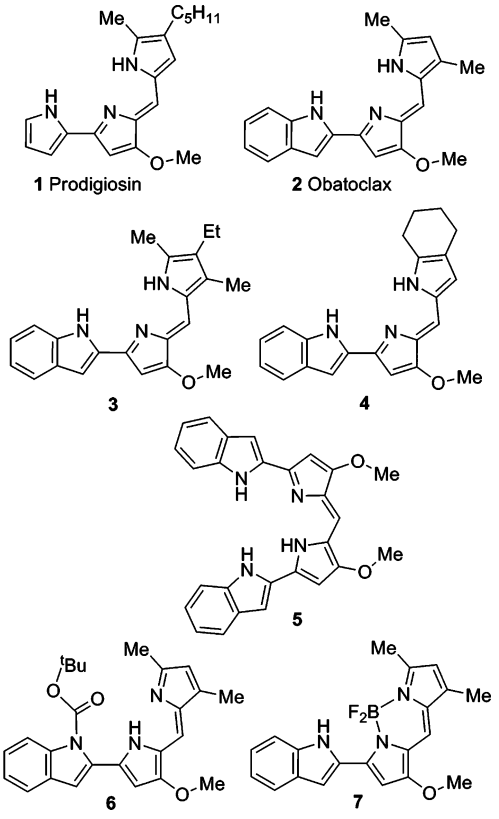
renal disease Bartter's syndrome, and some forms of myotonia.[5] Anion transport across lipid bilayers may also result in alterations of pH levels (by for example an HCl cotransport mechanism or a chloride/bicarbonate antiport process).[6,7] Since acidification of cytoplasm is an early event in apoptosis, it has been suggested that compounds that can change internal pH regulation can offer a possible approach to anticancer therapy.[8,9] In this Account, we cover work from the Burgos and Southampton groups on synthetic anion transporters and their interaction with human cancer cell lines studied in Barcelona. In general, we have shown that the most effective anion transporters across a number of different transporter systems have the greatest anticancer activity.

2. Transporters Based on Natural Products

Prodigiosin **1** is a red pigment produced by several bacteria strains including *Serratia marcesens*. This alkaloid belongs to a family of compounds sharing a common 4-methoxy-2,2′-bipyrrolpyrromethene structure called prodiginines.[10] These compounds have been extensively studied in the last 20 years because of their pharmacological properties, including anticancer and immunosuppressive activity.[11,12] There may be a number of mechanisms responsible for these useful properties, and several have been proposed. Among these, the ionophoric activity of these compounds was recognized early, and consequently prodigiosin is the most studied natural product facilitating the transmembrane transport of anions.[13,14] Both HCl cotransport and chloride/bicarbonate exchange are promoted by these compounds. The interest in the prodiginines is not only academic; a synthetic prodiginine named obatoclax **2** is currently in clinical trials and shows promise as a future treatment for a number of different cancers.[15] We decided to study the anion transport properties of this compound together with other synthetic analogues and to investigate whether the anionophoric activity is correlated to the cytotoxicity of these derivatives.[16] In order to address these goals, the Burgos group synthesized obatoclax **2** along with the similar analogues **3** and **4**. In order to manipulate the number of hydrogen bond donors and thus the anion coordination properties of these compounds derivatives **5**–**7** were also included in this study. Compound **6** is an obatoclax analogue containing a N-Boc protected indole group, thus this group is not available for anion binding and it would induce steric hindrance at the binding pocket of the dipyrromethene moiety. Compound **7** is a BODIPY derivative and in this case the dipyrromethene moiety is not available for anion coordination.



The solid state structures of some of these compounds (Figure 1), along with titration experiments in solution and a complete theoretical study of the interaction of **2** with anions in different solvents, indicate the ability of these compounds to interact effectively with anions through hydrogen bonds.[16]

Anion transport experiments showed that some of these compounds were extremely efficient anion transporters. Chloride release from POPC liposomes mediated by these compounds was monitored using chloride selective electrodes. Using Hill plot analyses, the concentration of carrier needed to achieve a 50% release of encapsulated chloride (EC_{50}) was calculated. This parameter allows a straightforward comparison of the relative efficiency of the different compounds as anion exchangers (Table 1). In the bicarbonate/chloride exchange assay the relative EC_{50} of these derivatives was found to follow the trend **3** < **2** < **4** < **5** ≪ **6**. The BODIPY analogue **7** was found to be essentially inactive as an anion transporter. Varying the external composition of the aqueous solution in which these experiments are carried out resulted in dramatic differences in chloride efflux rates. Thus, using lipophilic nitrate as the external

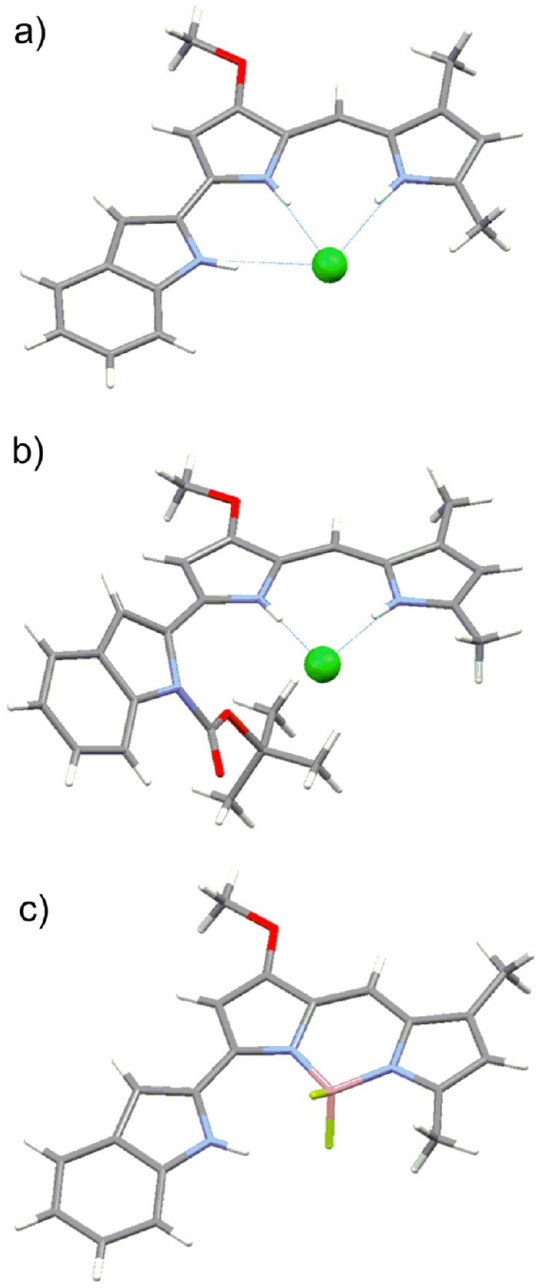


FIGURE 1. X-ray crystal structures of (a) compound **2**·HCl, (b) compound **6**·HCl, and (c) compound **7**.

TABLE 1. EC_{50} Values and IC_{50} Values of Compounds **2**–**6**

		IC_{50} [μM][b]		
compd	$EC_{50,290s}$ [μM][a]	24 h	48 h	72 h
2	0.28 ± 0.01	1.61 ± 0.15	0.25 ± 0.54	0.13 ± 0.10
3	0.18 ± 0.02	1.02 ± 0.21	0.29 ± 0.07	0.23 ± 0.06
4	0.75 ± 0.04	0.96 ± 0.05	0.42 ± 0.19	0.32 ± 0.02
5	2.10 ± 0.25	0.97 ± 0.12	0.60 ± 0.05	0.61 ± 0.13
6	18.90 ± 3.13		2.01 ± 1.08	1.68 ± 0.11

[a]Concentration needed to achieve 50% of chloride efflux after 290 s for the bicarbonate antiport process. [b]Values obtained from MTT assay on GLC4 cell line at 24, 48, and 72 h exposure time. Results depicted represent a mean of three independent experiments with standard deviation.

anion resulted in an enhanced chloride efflux whereas using sulfate resulted in no significant chloride release. These results supported anion exchange as the main mechanism of action accounting for the transmembrane transport activity displayed by these compounds. Moreover direct evidence of transmembrane bicarbonate transport mediated by these compounds was obtained from ^{13}C NMR experiments using ^{13}C labeled bicarbonate.[7] Figure 2 shows a representative experiment using an active bicarbonate carrier. At the beginning of the experiment (Figure 2a) two different signals corresponding to the encapsulated and extravesicular bicarbonate can be seen. After addition of the active carrier and a chloride pulse bicarbonate efflux occurred and just a signal corresponding to the extravesicular bicarbonate can be seen in the ^{13}C NMR (Figure 2b). The addition of paramagnetic manganese(II) resulted in a broadening of this signal into the baseline, confirming the extravesicular location of the bicarbonate (Figure 2c).

The in vitro cytotoxicity of these compounds was evaluated on the small cell lung cancer cell line GLC4 in Barcelona (Table 1). Obatoclax **2** is in advanced clinical trials for the treatment of this condition; therefore, this cell line was chosen as a model. The first indication of the relationship between anion transport and cytotoxicity of these compounds came from the results of the BODIPY analogue **7**. This compound, structurally very similar to obatoclax, was found to be inactive as an anion transporter and to be essentially noncytotoxic. A relationship between the cytotoxicity and anion transport efficiency of the other compounds was evident. A plot of the values of $-\log IC_{50}$ and $-\log EC_{50}$ illustrates this correlation (Figure 3). These results support the idea of the relevance of the ionophoric activity of these compounds in their mode of action concerning the anticancer activity.

We next investigated the marine alkaloids tambjamines.[17,18] These natural compounds are structurally related to the prodiginines, sharing with those the 4-methoxy-2,2′-bipyrrole unit which, in this case, is decorated with an enamine substituent. They also possess useful biological activity, including in vitro antitumor activity.[19,20] We envisaged that these easy to make compounds could be useful in order to prepare libraries of compounds aimed to shed light into the different variables involved in the design of anion transporters and also to identify compounds with optimized anion transport properties and potential as anticancer chemotherapeutics. We first investigated the anion transport properties of a set of these compounds in model phospholipid liposomes. We chose four naturally occurring

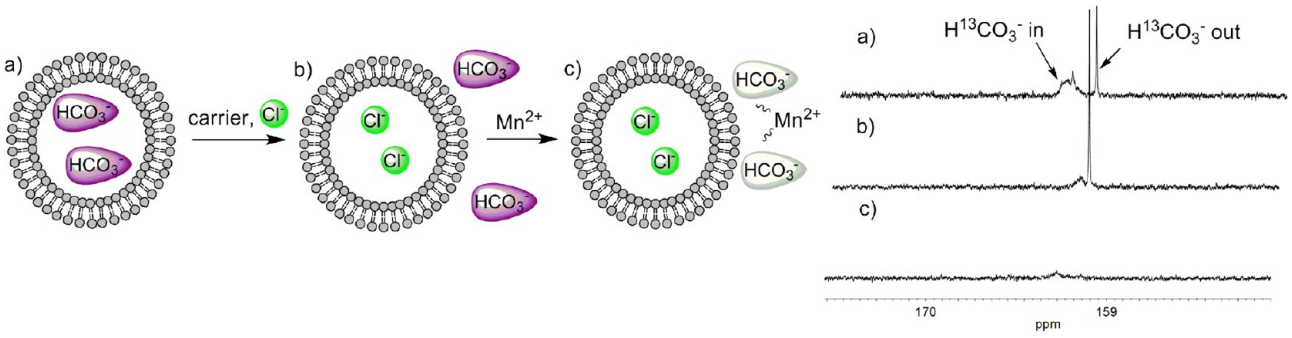


FIGURE 2. Representation of the ^{13}C NMR experiment sequence evidencing bicarbonate transmembrane transport facilitated by the pyrrole-based transporters.

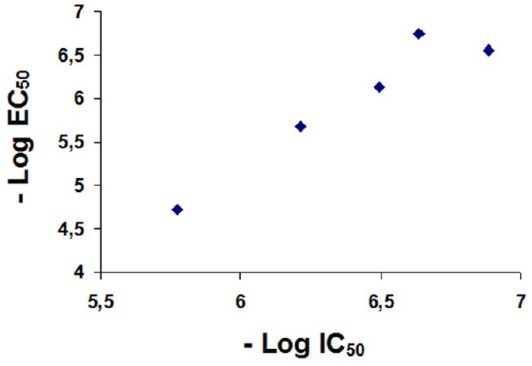
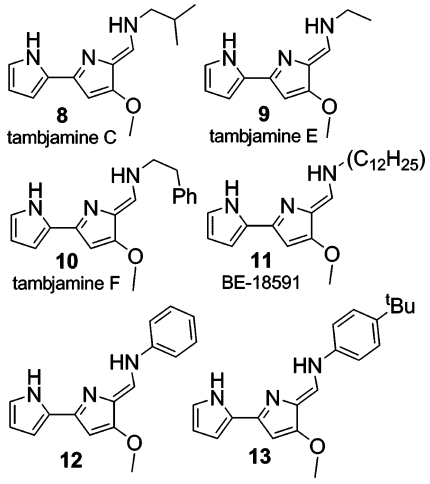


FIGURE 3. Representation of $-\log EC_{50}$ versus $-\log IC_{50}$ values of compounds **2**–**6** showing a linear correlation.

derivatives **8**–**11** and two synthetic, aryl substituted tambjamine analogues **12**, **13**.[21]



The chloride efflux from chloride loaded POPC vesicles facilitated by these derivatives was monitored using a chloride selective electrode under different conditions (Table 2). The chloride release efficiency is expressed as percentage of chloride release per second (% s^{-1}).[22] When there is only sulfate in the external medium very little release of chloride was observed (Table 2, *a*). On the other hand chloride release is switched on upon the addition of bicarbonate to the external medium (Table 2, *b*). Similar chloride efflux rates were observed when nitrate was used as the external medium using a 10-fold lower concentration of carrier (Table 2, *c*). The presence of a pH gradient had little effect on the transport results (Table 2, *d* and *e*). These results strongly support anion exchange as the mechanism of transport accounting for the anion transport promoted by these compounds. The lack of HCl cotransport is presumably due to the relative high basicity of the tambjamine derivatives. At physiological pH, these compounds do not deprotonate to a significant extent.

TABLE 2. Transport Activities (% s^{-1}) of Compounds **8**–**13**

compd	*a*	*b*	*c*	*d*	*e*
8	0.02	0.20	0.28	0.01	0.12
9	0.01	0.07	0.06	0.03	0.04
10	0.03	0.24	0.18	n.d.	0.14
11	0.03	0.51	0.34	n.d.	0.38
12	0.03	0.41	0.44	0.02	0.30
13	0.03	0.61	0.52	0.08	0.66
blank	0.00	0.01	0.00	0.00	0.00

[a]Vesicles loaded with 489 mM NaCl dispersed in 162 mM Na_2SO_4 (pH 7.2, 20 mM phosphate buffer). [b]Vesicles loaded with 489 mM NaCl dispersed in 162 mM Na_2SO_4 (pH 7.2, 20 mM phosphate buffer) upon addition of a $NaHCO_3$ pulse to make the extravesicular bicarbonate concentration 40 mM. [c]Vesicles loaded with 489 mM NaCl dispersed in 489 mM $NaNO_3$ (pH 7.2 with 5 mM phosphate buffer). [d]Vesicles loaded with 489 mM NaCl (pH 5.3, 20 mM MES buffer) dispersed in 162 mM Na_2SO_4 (pH 7.2, 20 mM MES buffer). [e]Vesicles loaded with 489 mM NaCl (pH 5.3, 20 mM MES buffer) dispersed in 162 mM Na_2SO_4 (pH 7.2, 20 mM MES buffer) upon addition of a $NaHCO_3$ pulse to make the extravesicular bicarbonate concentration 40 mM.

Using acridine orange staining, we demonstrated that these compounds also deacidified acidic organelles in GLC4 cancer cells (Figure 4). Moreover, ineffective anion carrier **9** was not able to deacidify the organelles, demonstrating that the anion transport experiments in vesicles were useful in order to predict in vitro activity of these compounds.

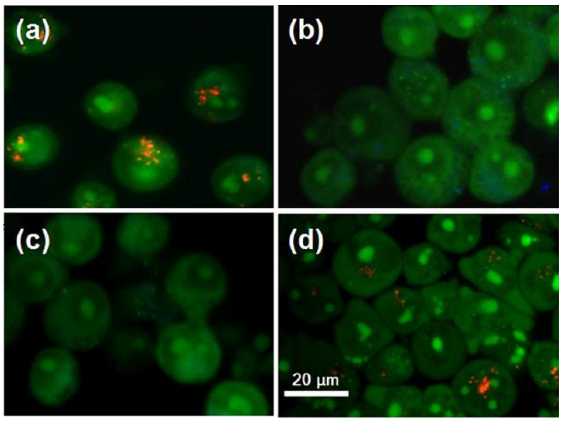
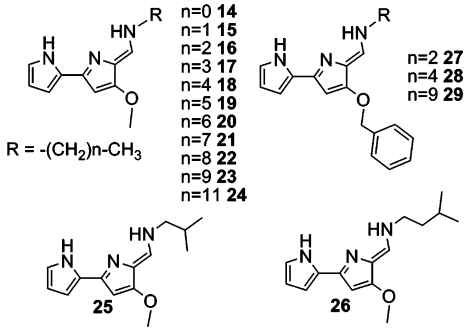


FIGURE 4. Acridine orange staining of small cell lung cancer line (GLC4) cells after exposure of 1 h to compounds **9**, **11**, and **13** (8 μM): (a) untreated cells (control), (b) cells treated with **11**, (c) cells treated with receptor **13**, and (d) cells treated with **9**. Untreated cells (a) or cells treated with **9** (d) showed granular orange fluorescence in the cytoplasm. Cells treated with compounds **11** and **13** (b, c) showed complete disappearance of orange fluorescence indicating deacidification of acidic organelles.

We next examined lipophilicity as an important parameter contributing to the anion transporter efficiency.[23] Until recently the role of lipophilicity as a factor involved in transporter design has not been considered in detail.[24] Therefore, we prepared a series of tambjamine derivatives **14**–**29** in which the enamine group presented alkyl-chains of different lengths. These compounds have similar polar surface areas and binding affinities. The log *P* values for these compounds were calculated using VCCLab software as an average value of log *P* values calculated using different structure and property-based methods.[25] These calculated values showed good correlation with the retention times observed for these compounds in reverse phase HPLC experiments, an experimental measure of the lipophilicity of the molecules.



The anion transport activity of these compounds was evaluated by monitoring chloride efflux from POPC vesicles in the bicarbonate/chloride exchange assay. For comparative purposes this activity was expressed as the initial rate of chloride efflux.[22] A plot of this activity versus log *P* is shown in Figure 5. The results highlight dramatic differences in anion transport efficiency as a result of variations of the length of the enamine alkyl chains. Compounds **14**–**16**, (R = Me, Et, Pr) are quite inactive. The transport efficiency gradually increased as the length of the chain increased, reaching a maximum with optimal activity. Once the lipophilicity of the derivatives is above the optimal range the transport efficiency diminishes again. We also demonstrated the usefulness of this concept in the design of new anion carriers. Changing the O–Me substituent in the central pyrrole ring of these derivatives to a O–Bn group is expected to have little impact on the total polar surface area and the binding affinity of these compounds. Yet this change results in an important change of lipophilicity compared to the parent –OMe substituted compounds.

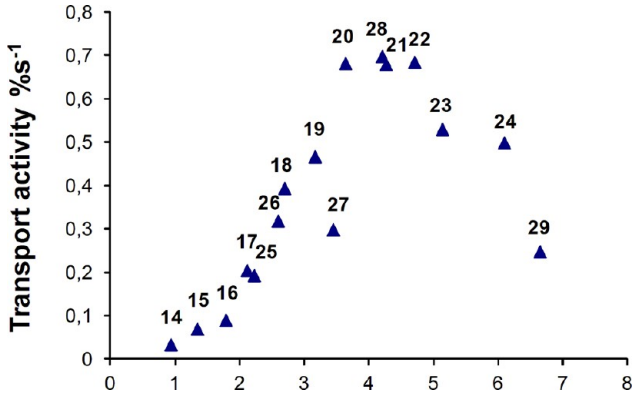


FIGURE 5. Representation of transport activity versus log *P* values of compounds **14**–**29**.

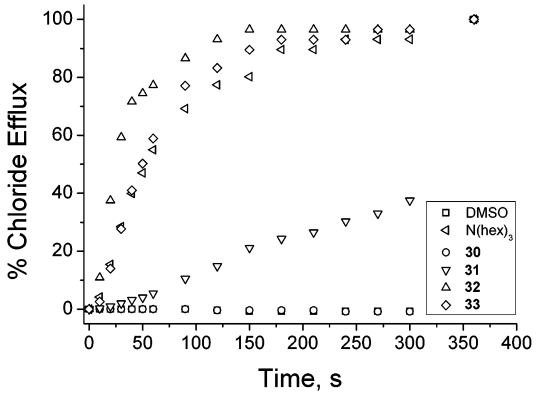


FIGURE 6. Chloride efflux promoted by 2% molar carrier to lipid concentrations of compounds **30**–**33** and trihexylamine ($N(hex)_3$) from unilamellar POPC vesicles loaded with 489 mM NaCl buffered to pH 7.2 with sodium phosphate salts. The vesicles were dispersed in 489 mM $NaNO_3$ buffered to pH 7.2 with 5 mM sodium phosphate salts. At the end of the experiment, detergent was added to lyse the vesicles and calibrate the ISE to 100% chloride release.

Using lipophilicity calculations, three derivatives **27**–**29** were synthesized. The anion transport activity found for these derivatives matched that anticipated from their lipophilicities, with optimal activity found for compound **28** with a log *P* value within the optimal range and lower activities for compounds **27** and **29** with log *P* values below and above the optimal range. These results underscore the importance of lipophilicity as a factor influencing the anion transport activity of molecular carriers and supported the idea of using these calculations to predict the activity of newly designed compounds within series of transporters.

TABLE 3. Association Constants K_a (M^{-1}) for the Binding of Compounds **31** and **33**–**41** to Various Anions in DMSO-d_6 Containing 0.5% Water at 298 K, Following the Most Upfield (Thio)urea NH[a,b]

	Cl^-	SO_4^{2-}	$H_2PO_4^-$	HCO_3^-	NO_3^-
			urea based compounds		
31	882[c]	>10^4[c]	443[d]	_[c,e]	_[f]
34	575	>10^4	452[d]	365	_[f]
35	166	>10^4[g]	>10^4[g]	>10^4[g,h]	_[fg]
36	405	>10^4	243[d]	156	_[f]
37	517	>10^4	_[i]	_[j]	_[f]
			thiourea based compounds		
33	191[c]	>10^4[c]	256[d]	_[c,j]	_[c,f]
38	179	>10^4	227[d]	_[j]	_[f]
39	128	>10^4	130	_[j]	_[f]
40	156	_[e]	_[i]	_[j]	_[f]
41	_[e]	_[e]	_[i]	_[j]	_[f]

[a]Errors are within 15%. [b]Anions added as TBA salts, except HCO_3^- which was added as a TEA salt. Fitted to 1:1 model. [c]Previously published data.[27] [d]Data for DMSO-d_6/10% water; data for DMSO-d_6/0.5% water could not be fitted. [e]Data could not be fitted to any model. [f]No significant shift of NH peaks, no binding. [g]Data for neat DMSO-d_6 by Ghosh and Ravikumar;[29] values for DMSO-d_6/0.5% water are expected to be lower. [h]2:1 model. [i]New peaks due to deprotonation of bound $H_2PO_4^-$ and subsequent binding of HPO_4^{2-}. [j]Peak broadening.

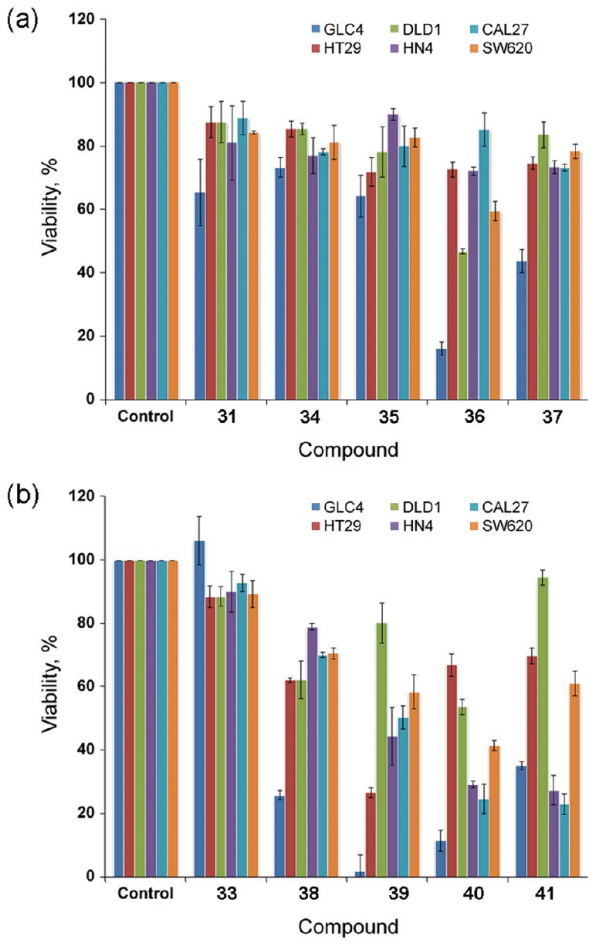


FIGURE 7. Single point screening of receptors **31** and **33**–**41** (10 μM) tested on a collection of different cancer cell lines, from left to right: GLC4, HT29, DLD1, HN4, CAL27, and SW620. (a) 24 h cell viability of cell exposure to ureas. (b) 24 h cell viability of cell exposure to thioureas.

TABLE 4. Overview of Transport Assays and Lipophilicity of Compounds **31** and **33**–**41**

compd	clogP[a]	clogP[b]	k_{ini}[c] (Cl^-/NO_3^-)	$EC_{50,270s}$[d] (Cl^-/NO_3^-)	n[e] (Cl^-/NO_3^-)	k_{ini}[f] (Cl^-/HCO_3^-)	$EC_{50,270s}$[d] (Cl^-/HCO_3^-)	n[e] (Cl^-/HCO_3^-)
				urea based compounds				
31	2.06	3.75	0.081	5.6	1.2	0.024	>5[g]	_[g]
34	2.53	4.98	0.571	0.43	1.4	0.081	>5[g]	_[g]
35	4.43	9.91	1.84	0.24	1.4	0.250	_[h]	_[h]
36	4.82	6.81	1.35	0.052	1.1	0.46	0.24	1.2
37	7.59	9.87	1.01	0.0044	1.6	0.77	0.036	1.5
				thiourea based compounds				
33	5.50	4.25	0.97	0.31	1.9	0.186	2.3	1.0
38	5.97	5.54	3.3	0.042	2.9	0.47	0.35	1.2
39	7.87	10.40	3.2	0.032	2.4	0.38	_[h]	_[h]
40	8.26	7.30	1.18	0.077	4.8	0.47	0.11	4.8
41	11.03	10.36	0.90	0.042	5.0	0.76	0.14	3.8

[a]clogP calculated using Spartan '08 for Macintosh (Ghose–Crippen model). [b]clogP calculated using Fieldview Version 2.0.2 for Macintosh (Wildman–Crippen model). [c]Initial rate of chloride efflux for 2% molar percentage carrier to lipid (% s^{-1}). [d]$EC_{50,270s}$ defined as concentration (mol % carrier to lipid) needed to obtain 50% efflux after 270 s. [e]Hill coefficient. [f]Initial rate of chloride efflux (after addition of $NaHCO_3$) for 2% carrier to lipid (% s^{-1}). [g]Accurate Hill analysis could not be performed due to low activity. [h]Meaningful Hill analysis could not be performed due to significant background transport in the absence of $NaHCO_3$ (HCl symport and/or Cl^-/SO_4^{2-} antiport).

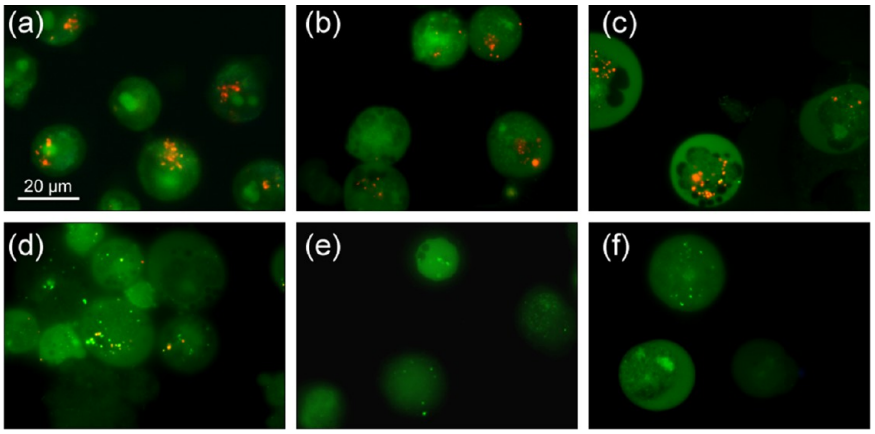


FIGURE 8. Acridine Orange staining of GLC4 cell line after exposure of 1 h to different receptors: (a) untreated cells (control), (b) cells treated with receptor **34**, (c) cells treated with receptor **35**, (d) cells treated with receptor **38**, (e) cells treated with receptor **39**, and (f) cells treated with receptor **40**. Panels (a)–(c) show cells with granular orange fluorescence in the cytoplasm, while panels (d)–(f) show complete disappearance of orange fluorescence cytoplasm granules.

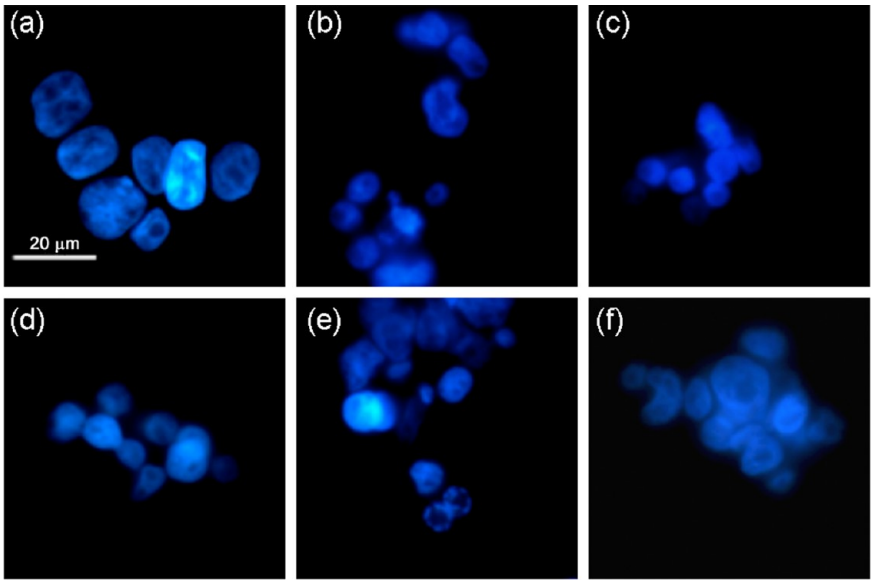
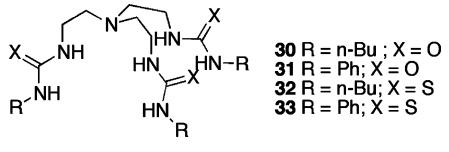


FIGURE 9. Hoechst 33342 staining of GLC4 cell line after exposure for 24 h to different receptors: (a) untreated cells (control), (b) cells treated with receptor **34**, (c) cells treated with receptor **36**, (d) Cells treated with receptor **38**, (e) cells treated with receptor **39**, and (f) cells treated with receptor **40**. Panels (a)–(c) show cells with normal nuclear morphology, while panels (d)–(f) show condensation of the nuclei and nuclei with "bean shape".

3. Synthetic Supramolecular Transporters

The Southampton group has developed several series of hydrogen bond donor anion transporters capable of both chloride and bicarbonate transport.[26] In 2010, we decided to investigate the transport properties of simple tren-based trisureas and thioureas **30**–**33**.[27] Analogous tren-based systems had previously been investigated for their anion and particularly oxo-anion complexation properties by Custelcean et al.,[28] Ghosh and Ravikumar,[29] and Wu et al.[30] We found that in POPC vesicles simple tren-based tris-thioureas **32** and **33** were effective chloride/nitrate and chloride/bicarbonate antiporters whereas urea analogues **30** and **31** had significantly reduced or no anion transport activity (Figure 6).



30 R = n-Bu ; X = O
31 R = Ph; X = O
32 R = n-Bu; X = S
33 R = Ph; X = S

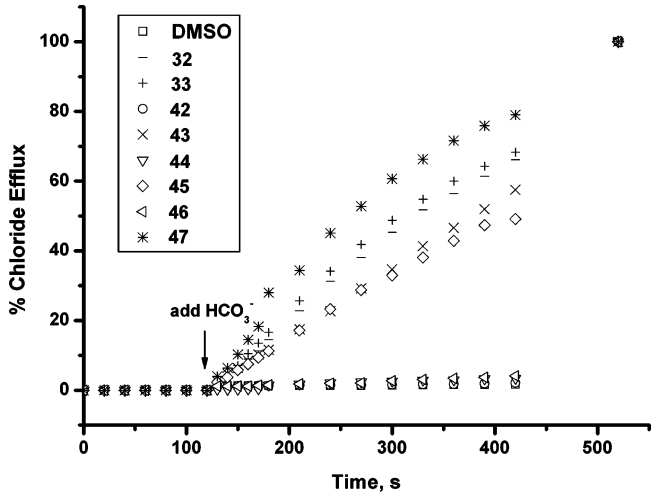
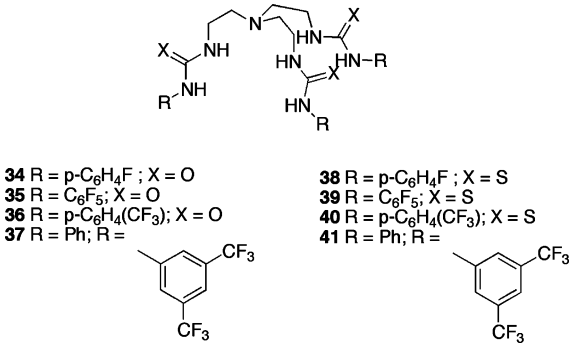


FIGURE 10. Chloride efflux promoted by 0.02 molar equiv of receptors **32**, **33**, and **42**–**47** from unilamellar POPC vesicles loaded with 489 mM NaCl buffered to pH 7.2 with 20 mM sodium phosphate salts upon addition of a $NaHCO_3$ pulse to make the extravesicular bicarbonate concentration 40 mM. The vesicles were dispersed in 167 mM Na_2SO_4 buffered at pH 7.2 with 20 mM sodium phosphate salts. At the end of the experiment, detergent was added to lyse the vesicles and calibrate the ISE to 100% chloride release.

For applications in biological systems, we wished to produce transporters that would achieve high ion flux at low carrier concentration. A common strategy in medicinal chemistry to improve the physiological properties of compounds is to synthesize fluorinated analogues. Fluorinated compounds often possess higher metabolic stability and may be less toxic and than their unfluorinated analogues.[31] Furthermore, fluorination of aromatic compounds results in increased lipophilicity and hydrogen bond acidity, which may subsequently lead to enhanced anion binding properties.[32] Both characteristics are expected to be favorable for the transport of anions across a lipid bilayer.



Proton NMR titration techniques were used to elucidate the stability constants of this series of receptors with biologically relevant anions in DMSO-d_6/0.5% water solution (Table 3). Vesicle anion transport studies of these systems using chloride selective electrodes demonstrated that the fluorinated compounds were significantly more effective transporters than the nonfluorinated analogues as illustrated by the EC_{50} values from Hill analysis studies obtained for the compounds for both chloride/nitrate and chloride/bicarbonate antiport (Table 4).[33] We propose that a predominant factor for the enhanced activity of the fluorinated compounds is their enhanced lipophilicity although other molecular parameters may also affect the propensity of these systems to transport anions. Interestingly there was also evidence for HCl cotransport and an unusual Cl^-/SO_4^{2-} antiport process with some of the fluorinated derivatives.

TABLE 5. Stability Constants K_a (M^{-1}) for Compounds **48**–**59** with Chloride and Nitrate (added as tetrabutylammonium salts) and Bicarbonate (added as the tetraethylammonium salt) in DMSO-d_6/0.5% Water at 298 K[a]

	chloride	bicarbonate	nitrate
48	64[b]	2330	[c]
49	17	414	[c]
50	154	$>10^4$	[c]
51	40	2150[d]	[c]
52	95	3860	[c]
53	25	[e]	[c]
54	101	4050	[c]
55	26	[e]	[c]
56	16	121	[c]
57	14	262	[c]
58	23	329	[c]
59	26	931[f]	[c]

[a]All errors <15%. Data fitted to a 1:1 binding model. Binding constant obtained by following most upfield NH unless stated otherwise. [b]Binding constant obtained by following downfield urea NH due to peak overlap. [c]No significant interaction observed. [d]Binding constant obtained by following indole NH due to broadening of urea NH signals. [e]Sigmoidal curve could not be fitted to a suitable binding model. [f]Binding constant obtained by following aromatic CH due to significant broadening of thiourea NHs.

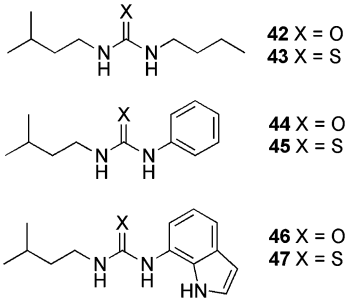
The anion transport activity of tripodal ureas and thioureas prompted us to investigate the in vitro bioactivity of these receptors on tumor cell lines by the evaluation of cell viability, estimation of the pH changes in acidic cell compartments by Acridine Orange staining and by nuclear Hoechst staining for the assessment of apoptotic cell death induction. Cell viability studies (Figure 7) showed that the most effective transporters reduced the viability of a range of human cancer lines, most notably GLC4 (small cell lung cancer). Acridine orange staining showed that these transporters were capable of depolarizing acidic compartments within GLC4 cells (Figure 8) while Hoechst staining of GLC4 demonstrated that the same transporters trigger apoptosis of these cells (Figure 9).

TABLE 6. Overview of Transport Assays and Lipophilicity of Compounds **48**–**59**

	clogP[a]	clogP[b]	$EC_{50,270s}$[c] (Cl^-/NO_3^-)	n[d] (Cl^-/NO_3^-)	$EC_{50,270s}$[c] (Cl^-/HCO_3^-)	n[d] (Cl^-/HCO_3^-)
48	0.34	3.87	[e]	[e]	[e]	[e]
49	1.49	4.03	0.029	0.8	0.18	0.7
50	1.90	6.23	0.47	1.3	1.2	2.0
51	3.04	6.40	0.016[f]	1.7[f]	0.081[f]	1.5[f]
52	−0.07	3.81	[e]	[e]	[e]	[e]
53	1.07	3.98	[e]	[e]	[e]	[e]
54	0.85	4.83	1.1	1.6	[e]	[e]
55	2.00	5.00	2.6	1.2	[e]	[e]
56	1.74	3.39	[e]	[e]	[e]	[e]
57	2.89	3.55	2.9	1.4	3.1	0.6
58	2.35	4.41	1.4	2.4	[e]	[e]
59	3.50	4.57	0.44	1.6	0.59	1.1

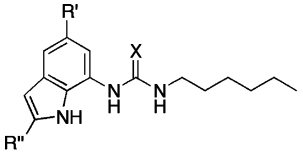
[a]clogP calculated using Spartan '10 for Macintosh (Ghose–Crippen model). [b]clogP calculated using Fieldview 2.0.2 for Macintosh (Wildman–Crippen model). [c]$EC_{50,270s}$ defined as concentration (mol % carrier to lipid) needed to obtain 50% efflux after 270s. [d]Hill coefficient. [e]Accurate Hill analysis could not be performed due to low activity. [f]Some of the observed activity is from H^+/Cl^- cotransport.

In order to maximize the chances that the transporters developed in the Southampton group have acceptable ADME (absorption, distribution, metabolism and excretion) properties we decided to investigate whether we could develop systems that conform to Lipinski's rule of five.[34] We therefore wished to develop smaller transporters and hence we examined the transport properties of simple molecules containing a thiourea group (**43**, **45**, **47**) and urea groups (**42**, **44**, **46**).[35] The thiourea compounds were found to be capable of chloride/bicarbonate antiport (Figure 10), with compound **47**, which contains an indole group linked to the thiourea via the 7-position, showing the fastest rate of chloride/bicarbonate antiport among this series.

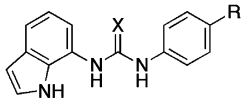


42 X = O
43 X = S
44 X = O
45 X = S
46 X = O
47 X = S

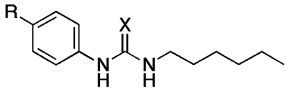
Compound **47** proved to be a very effective chloride/nitrate and chloride/bicarbonate antiporter. We therefore conducted a study on a range of similar urea and thiourea compounds with and without appended trifluoromethyl groups **48**–**59**.[36] The stability constants of these compounds measured in DMSO-d_6/0.5% water which revealed generally moderate affinities for chloride across the series and stronger interactions with bicarbonate (Table 5). Transport experiments with chloride containing vesicles revealed a range of activity (Table 6). In general, the trifluoromethyl-functionalized receptors had greater anion transport activity than their nontrifluoromethyl functionalized analogues and once again thioureas are more active than ureas. The results of chloride/nitrate exchange experiments are shown in Figure 11. The *in vitro* cytotoxic activity of receptors **48**–**59** was assessed on several cancer cell lines including human small-cell lung carcinoma (SCLC) GLC4, human melanoma A375, human colon adenocarcinoma SW480, and human oral adenosquamous carcinoma CAL27. A single-point screening at 10 μM was performed using the cell viability MTT assay in order to evaluate cytotoxic effects after receptor exposure (48 h). Receptors **50**, **51**, **54**, **58**, and **59** showed significant cytotoxicity, especially in GLC4 and A375 cells, while no significant effect was observed after treatment with the other receptors at the same dose and time.



48 X = O, R' = H, R" = H
49 X = S, R' = H, R" = H
50 X = O, R' = CF_3, R" = nBu
51 X = S, R' = CF_3, R" = nBu



52 X = O, R = H
53 X = S, R = H
54 X = O, R = CF_3
55 X = S, R = CF_3



56 X=O, R = H
57 X = S, R = H
58 X = O, R = CF_3
59 X = S, R = CF_3

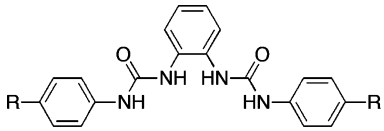
Dose–response curve experiments were performed, and IC_{50} values (inhibitory concentration of 50% of cell

population) were calculated for cytotoxic compounds in the most sensitive cancerous cell lines and also in a noncancerous human MCF10A mammary epithelial cell line (Table 7). These results corroborate the potency of these cytotoxic receptors, showing IC_{50} values around 10 μM in GLC-4 cells. We observe a significant reduction in cytotoxicity in MCF10A compared to cancerous cells, suggesting some cytotoxic specificity of these receptors toward cancerous cells. All the fluorinated compounds except compound **55** have cytotoxic activity but interestingly compound **55** has the lowest anion transport ability among the fluorinated receptors. Acridine Orange staining showed the cytotoxic compounds were again capable of depolarizing acidic compartments within A375 cells (while the other less active transporters did not) (Figure 12) and Hoechst staining of these cells demonstrated that the highly active transporters trigger apoptosis (Figure 13).

Similar experiments have been conducted on a series of compounds based on the *ortho*-phenylenediamine scaffold.

TABLE 7. IC_{50} Values (μM) of Cytotoxic Compounds **50**, **51**, **54**, **58**, and **59** on GLC4, A375 Cancerous Cell Lines and MCF10A Noncancerous Cell Line

	GLC4	A375	MCF10A
50	6.0 ± 1.1	17.4 ± 5.5	>70
51	6.3 ± 0.5	14.3 ± 0.1	>40
54	9.7 ± 1.7	22.9 ± 2.6	>200
58	16.8 ± 0.4	13.7 ± 2.0	>200
59	14.9 ± 0.1	21.1 ± 0.8	>30



60 R = CF_3
61 R = NO_2

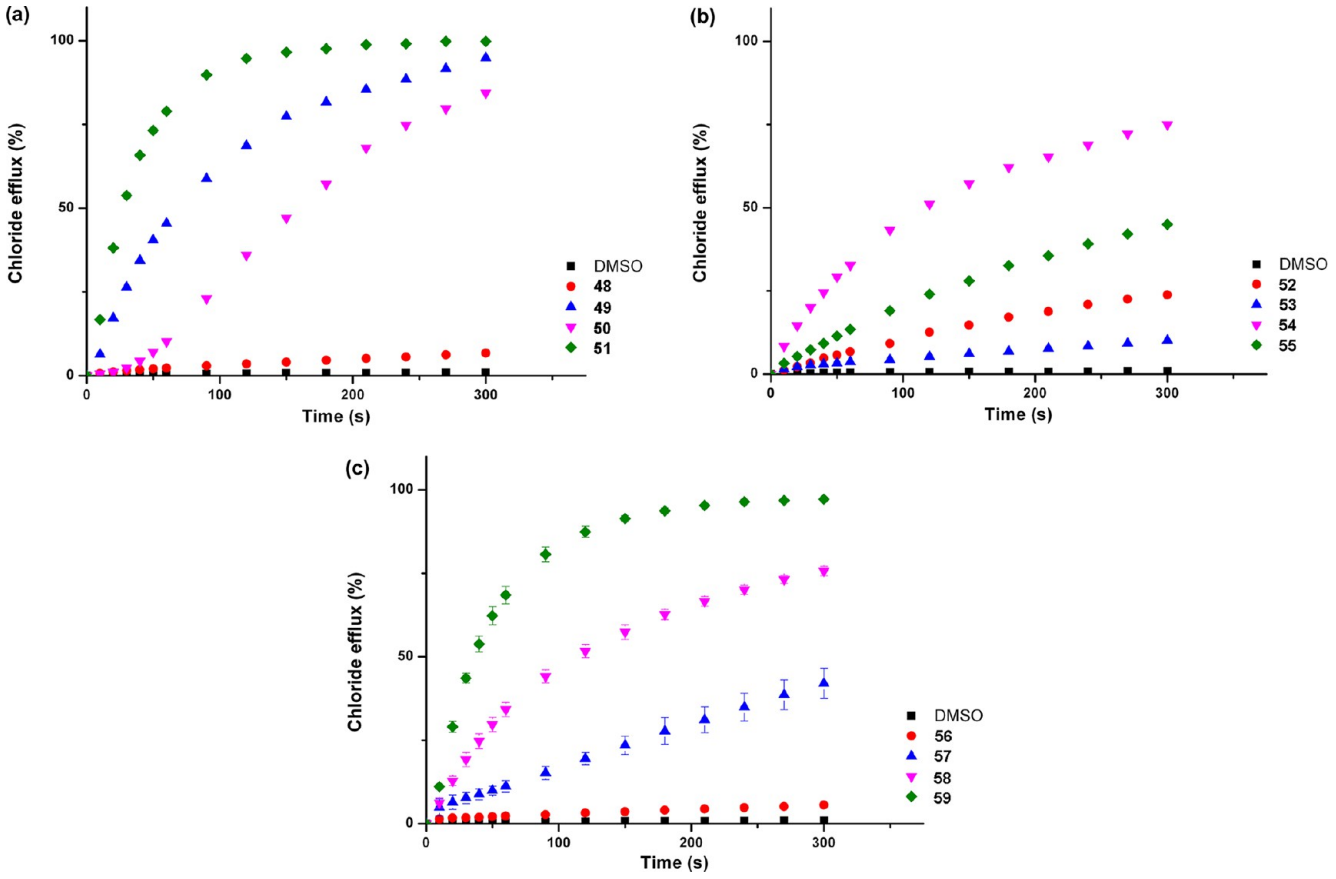


FIGURE 11. Chloride efflux promoted by a DMSO solution of compounds **48**–**59** (2 mol % carrier to lipid) from unilamellar POPC vesicles loaded with 488 mM NaCl buffered to pH 7.2 with 5 mM sodium phosphate salts. The vesicles were dispersed in 488 mM $NaNO_3$ buffered to pH 7.2 with 5 mM sodium phosphate salts. At the end of the experiment, detergent was added to lyse the vesicles and calibrate the ISE to 100% chloride efflux. Each point represents an average of three trials. DMSO was used as a control. Reproduced with permission from ref 36. Copyright 2012 Royal Society of Chemistry.

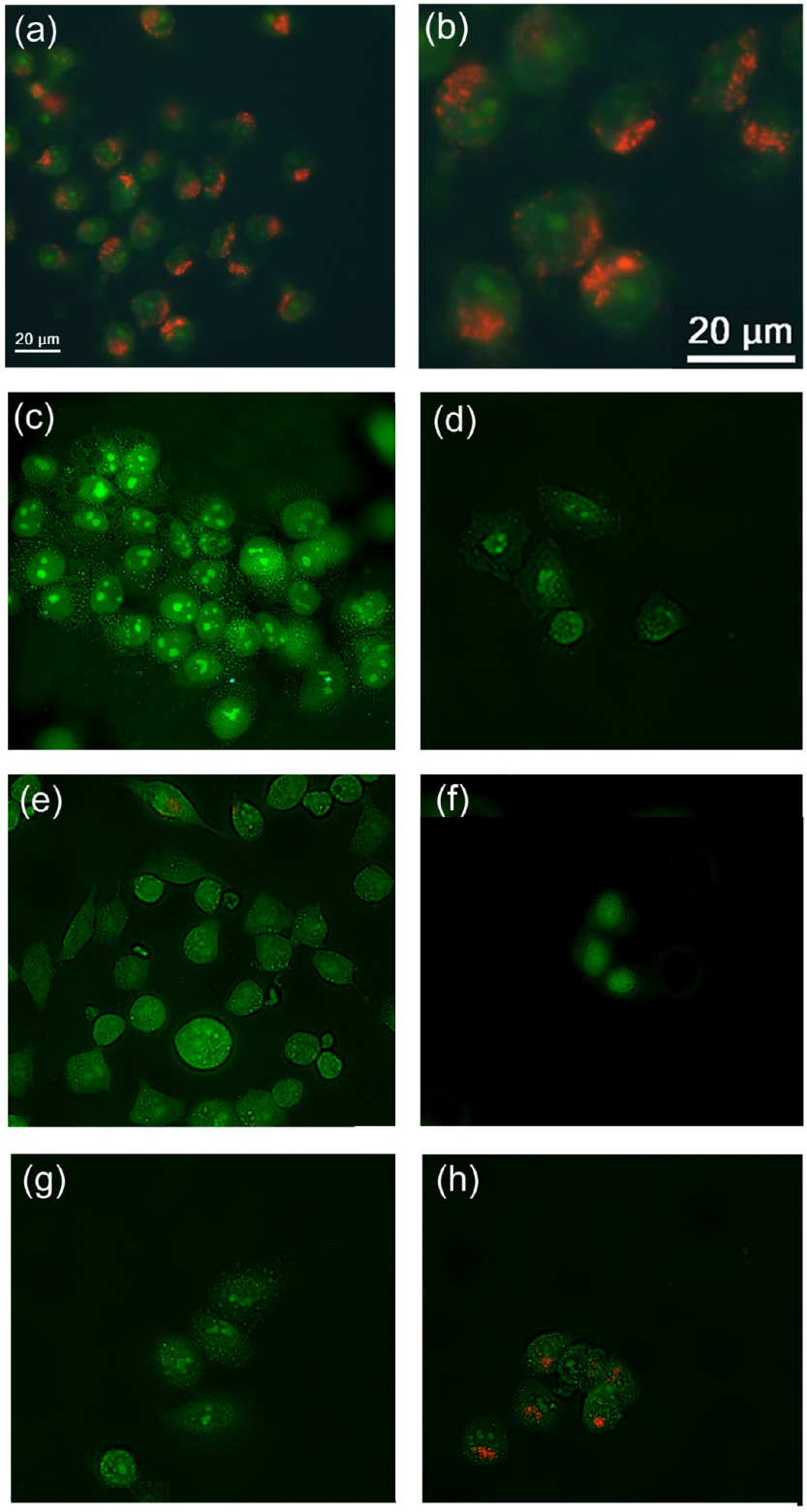


FIGURE 12. Acridine Orange staining of melanoma A375 cells after 1 h exposure to different compounds (10 μM): (a, b) untreated cells, (c) compound **50**, (d) compound **51**, (e) compound **54**, (f) compound **58**, (g) compound **59**, and (h) compound **52**. Cells with cytoplasmic granular orange fluorescence (a, b, h); cells with complete disappearance of cytoplasmic orange fluorescence (c–g). Reproduced with permission from ref 36. Copyright 2012 Royal Society of Chemistry.

Again active transporters such as **60** and **61** were found to reduce the viability of human cancer cell lines, to depolarize acidic compartments with A375 cells, and to trigger apoptosis in these cells.[37]

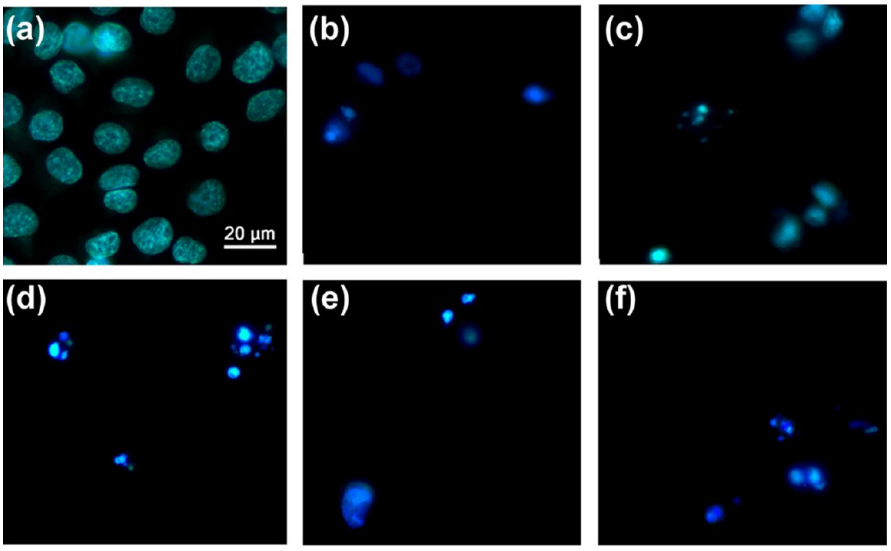


FIGURE 13. Hoechst 33342 staining of A375 cells after 48 h exposure to different receptors (10 μM): (a) untreated cells, (b) compound **50**, (c) compound **51**, (d) compound **54**, (e) compound **58**, and (f) compound **59**. Cells with typical nuclear morphology (a); cells with nuclear condensation and apoptotic bodies (b–f). Reproduced with permission from ref 36. Copyright 2012 Royal Society of Chemistry.

4. Conclusions

The results obtained show that active anion transporters are capable of depolarizing acidic compartments with cells so lowering pH_i. The active transporters also trigger apoptosis in a range of human cancer cell lines. Systems based on the structure of the natural product prodigiosin as well as purely synthetic supramolecular transporters have similar properties in these respects. More work must be done both to develop new transporters that work at lower concentrations and to understand the mechanism of depolarization of the acidic organelles within cells. This, we hope, will lead to a greater understanding of the processes triggered by these compounds in biological systems and the generation of new families of compounds with anticancer properties.

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Philip A. Gale was born in Liverpool, U.K. He received a B.A. (Honors) in chemistry in 1992, M.A. in 1995, and a D.Phil. in 1995 from the University of Oxford. He then moved as a Fulbright Postdoctoral Fellow to the University of Texas at Austin in 1995, returning to the University of Oxford in 1997 as a Royal Society University Research Fellow, and moving in 1999 to the University of Southampton, where he is now Professor of Supramolecular Chemistry and Head of Chemistry. A major focus of his research is the supramolecular chemistry of anionic species. He was won a number of research awards including the RSC's Corday-Morgan Medal and Prize.

Ricardo Pérez-Tomás was born in Murcia, Spain. He received his Ph.D. at the University of Murcia with Prof. José Ballesta and Prof. J. M. Polak on comparative neuroendocrine system. From 1989, he is a Professor of Cell Biology in the Department of Pathology and Experimental Therapeutic at the University of Barcelona, Spain. His main research focus is in the characterization of novel anticancer drugs and their mechanism of action as well as in assessing the therapeutic potential of these molecules in the treatment of cancer.

Roberto Quesada was born in Cangas de Onis, Asturias, Spain in 1974. He received his Ph.D. in 2002 with Professors Javier Ruiz and Prof. Víctor Riera at the University of Oviedo. After postdoctoral research at the Trinity College Dublin (Prof. Sylvia M. Draper, 2003) and the University of Southampton (Prof. Philip A. Gale, 2004), he was awarded with a Juan de la Cierva contract in the group of Prof. Pilar Prados at the Universidad Autónoma de Madrid in 2006. He moved to the University of Burgos as a Ramon y Cajal Fellow in 2008, being promoted to Assistant Professor in 2012. His research interests include synthetic chemistry and supramolecular chemistry in lipid bilayer membranes.

FOOTNOTES

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The authors declare no competing financial interest.

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