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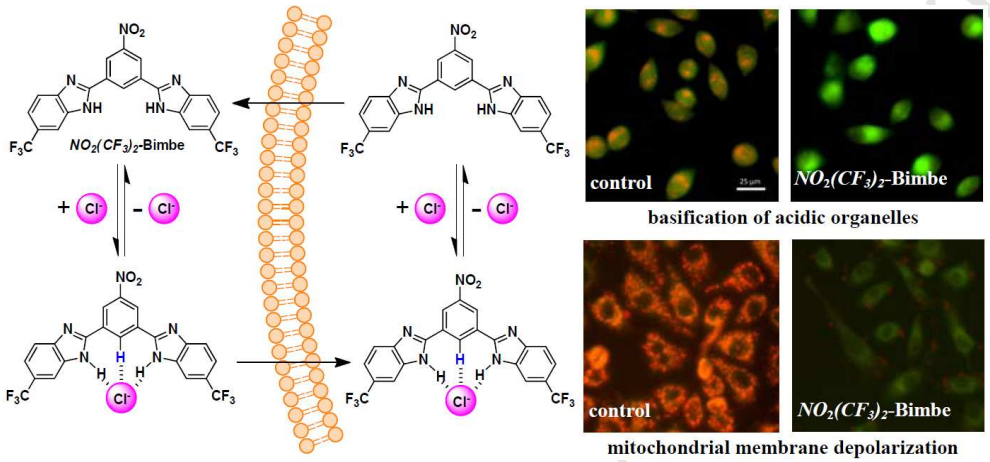
Graphical Abstract



A series of 1,3-bis(benzimidazol-2-yl)benzene derivatives were synthesized and found to exhibit potent chloride-mediated apoptosis-inducing activity toward the tested cancer cells.

Synthesis, anionophoric activity and apoptosis-inducing bioactivity of benzimidazolyl-based transmembrane anion transporters

Xi-Hui Yu, Chen-Chen Peng, Xiao-Xiao Sun and Wen-Hua Chen*

Guangdong Provincial Key Laboratory of New Drug Screening, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, P. R. China. E-mail: whchen@smu.edu.cn

Abstract: In this paper we show that a series of 1,3-bis(benzimidazol-2-yl)benzene (*m*-Bimbe) derivatives exhibit excellent performance as transmembrane anion transporters with anticancer activity. The transport efficiency of *m*-Bimbe and its derivatives has been firstly optimized by adding a strong electron-withdrawing nitro group at the 5-position of the central phenyl subunits to enhance the CH···anion interactions. Evidences for the interactions were obtained from ESI MS, spectrophotometric and ^{1}H NMR titrations. These compounds exhibit potent anionophoric activity in both liposomal models and live cells. In particular, the 5-nitrated derivatives having nitro or trifluoromethyl groups at the benzimidazoloyl subunits exhibit 2370- and 1721-fold enhanced anionophoric activity with the EC_{50} values as low as 36 and 50 nM, respectively. These compounds can disturb the cellular homeostasis of chloride anions, modify the intracellular pH and induce the basification of acidic organelles. Most of this series of *m*-Bimbe derivatives exhibit potent cytotoxicity toward the tested human solid tumor cell lines, and the 5-nitrated derivative bearing trifluoromethyl groups at the benzimidazoloyl subunits is the most active with the IC_{50} value in the low micromolar range. Mechanistic studies suggest that the transport of chloride anions across the cellular membranes plays a critical role in the cytotoxic effect and these compounds induce cell death probably *via* an apoptotic process.

Keywords: Anion transporter; Benzimidazole; Anionophoric activity; Cytotoxicity; Apoptosis

1. Introduction

During the past decades, considerable interest has been attracted in identifying small-molecular anion transporters that are capable of efficiently mediating the transport of anions across lipid membranes [1, 2]. By modulating the intracellular pH [3, 4], or disrupting the cellular ionic homeostasis in cancer or bacteria cells [5, 6], effective anion transporters are able to induce cell death and thereby may serve as a new therapy for cancers and bacterial infections [1, 7]. For example, Gale *et al* have shown that pyridine diamide-strapped calix[4]pyrroles are able to induce coupled chloride/sodium transport both in liposomal models and cells, and promote cell death by increasing the intracellular concentrations of chloride and sodium ions [8]. Recently, they have further shown that some squaramide-based anion transporters are able to disrupt the autophagy of cells and induce their apoptosis by perturbing the cellular chloride concentrations [9]. Talukdar *et al* have shown that some bis(sulfonamide) compounds are able to facilitate the transport of chloride anions across cellular membranes and disrupt the ionic homeostasis to impose cell death [6]. Schmitzer *et al* have described the strong antibacterial properties of some benzimidazolium-based anion transporters, which is considered as a result of their ability to insert into the cellular membranes and alter the membrane permeability for chloride anions [10]. Such chloride-mediated biological activity has driven forward the high potential applications of synthetic anion transporters in biomedical field [1, 7].

On the other hand, some inherent disadvantages of the anion transporters reported to date are also apparent. In particular, the high molecular weights and lipophilicity do not meet the requirements for drug-likeness [11]. Therefore, small-molecular organic compounds that have high anion transport activity and meanwhile fall within the rules of thumb, such as the Lipinski's rule of five, are particularly attractive from the viewpoint of new drug discovery [1, 2]. In the endeavor to optimize the efficiency of anion transporters, several strategies, such as

lipophilicity [4, 12], configuration [13-15] and flexibility [16], have been exploited. Because anions need to be desolvated and stabilized during crossing the hydrophobic phospholipid bilayer membranes [17], non-covalent interactions, such as electrostatic [18, 19], hydrogen bonding [20-22] and anion–π interactions [23-25] have been successfully utilized to design powerful anion transporters. Among these interactions, hydrogen bonding formed from conventional OH and NH donors is a predominant one [20-22]. In some examples, anion transport activity has been maximized due to the strong hydrogen-bonding interactions of anions with pre-organized donors [21, 22]. Recently, CH is recognized as a soft hydrogen-bond donor and therefore CH···anion interaction may serve as a promising alternative to achieve high binding affinity and efficient transmembrane anion transport [26, 27].

In these aspects, because of the ability to recognize anions *via* hydrogen-bonding interactions [28], imidazolyl or benzimidazolyl-based compounds represent a class of attractive molecular scaffolds and have been demonstrated to exhibit promising anion transport properties with potential bioactivity [10, 18, 19, 29-33]. In our recent studies we have shown that 1,3-bis(benzimidazol-2-yl)benzene (*m*-Bimbe, Fig. 1) exhibits efficient anion transport activity [34, 35]. Preliminary study on the structure-activity relationship, by using *m*-Bimbe, 1,2-bis(benzimidazol-2-yl)benzene (*o*-Bimbe), 1,4-bis(benzimidazol-2-yl)benzene (*p*-Bimbe), 2-phenylbenzimidazole (Imbe) and 1,3-bis(*N*-methylbenzimidazol-2-yl)benzene (Me_2bimbe) (Fig. 1), indicates that the spatial orientation of the two benzimidazolyl subunits at the central phenyl group greatly affects the anion transport activity and the benzimidazoloyl-*NH* motifs are required for maintaining such activity [35]. More interestingly, the activity may be greatly improved by adding strong electron-withdrawing substituents, for example, trifluoromethyl and nitro groups onto the benzimidazolyl subunits (to give $(CF_3)_2$-Bimbe and $(NO_2)_2$-Bimbe, respectively, Fig. 1) [35]. The transport activity increases with the electron-withdrawing strength of the substituents in the order of $NO_2 > CF_3 > F > Cl > Br > H$ [35]. These findings, together with the low molecular weight (310 Da), appropriate lipophilicity (*c*log*P* = 4.60) and

ready availability (from commercial sources or the condensation of *o*-phenylenediamine with isophthalic acid or isophthalaldehyde), has highlighted the potentials of *m*-Bimbe as a lead compound for the development of potent, drug-like anion transporters.

The objectives of the work described herein are two-fold, that is, to further optimize the anionophoric activity of *m*-Bimbe and to explore the potentials of *m*-Bimbe derivatives as anti-cancer agents that function *via* a mechanism of transmembrane anion transport. The study by Tiburcio *et al* has shown that *m*-Bimbe binds anions *via* the cooperative interactions with both benzimidazolyl-*NH* and $-C_2H$ donor motifs [36]. Our study has indicated that the enhanced anion transport activity of *m*-Bimbe derivatives bearing strong electron-withdrawing substituents at the benzimidazolyl subunits, may be ascribed to the increase in the acidity of the benzimidazolyl-*NH* motifs caused by the strong electron-withdrawing groups [35]. Therefore, the first objective is to further optimize the anion-binding affinity and transport activity by increasing the acidity of $-C_2H$. This may be achieved by adding a strong electron-withdrawing substituent to the central phenyl subunit in *m*-Bimbe. Thus, we nitrated *m*-Bimbe, $(CF_3)_2$-Bimbe and $(NO_2)_2$-Bimbe at the 5-position of the central phenyl subunits to give NO_2-Bimbe, $(NO_2)_3$-Bimbe and $NO_2(CF_3)_2$-Bimbe, respectively (Fig. 1). For comparison, we also synthesized the corresponding analogues of $(NO_2)_3$-Bimbe and $NO_2(CF_3)_2$-Bimbe, that is, $MeO(NO_2)_2$-Bimbe and $MeO(CF_3)_2$-Bimbe having an electron-donating 5-methoxy group.

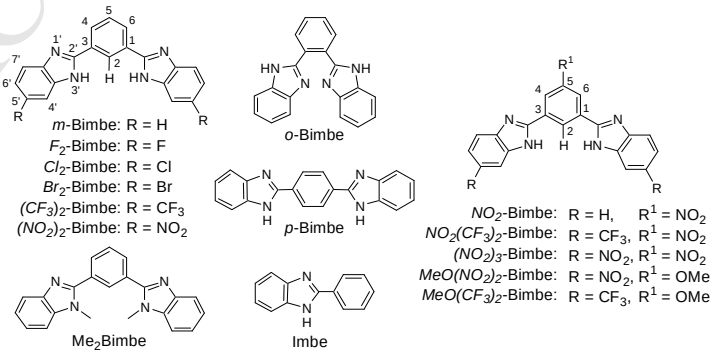
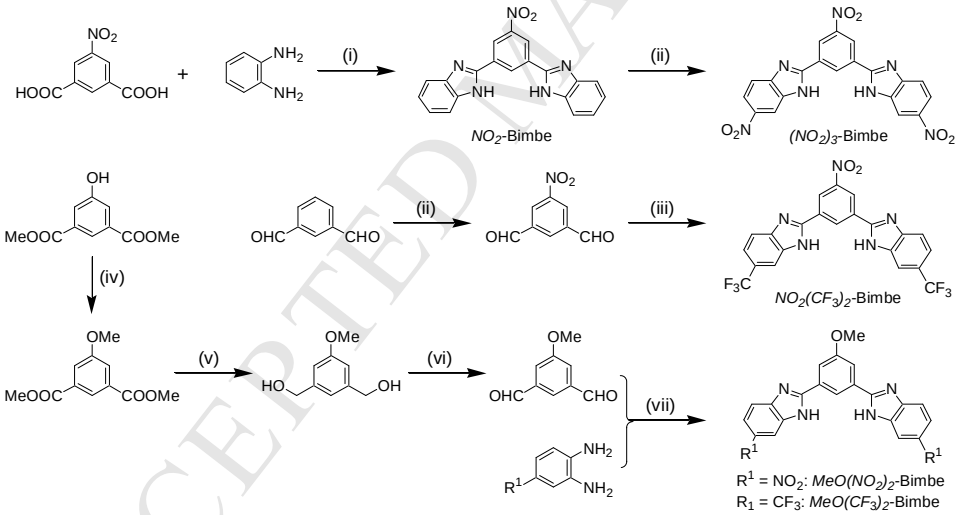


Fig. 1. Structures of *m*-Bimbe and its derivatives.

Secondly, we have shown in our recent study that *m*-Bimbe and its derivatives exhibit anion transport activity *via* a process of anion exchange with a minor level of proton/anion symport [34, 35], a mechanism of action that is similar to that of prodigiosin, a natural chloride carrier [37]. Considering the strong correlation between the proton/chloride symport transport rates and the *in vitro* cytotoxic activity of prodigiosin and its analogues [38], we are concerned about (1) whether *m*-Bimbe and its derivatives depicted in Fig. 1 exhibit anti-proliferative activity toward human solid tumor cells, (2) how the cytotoxicity (if there is any) relates to the anion transport across liposomal and cellular membranes and (3) what the probable mechanism of action is.

2. Results and discussion

2.1 Chemistry



Scheme 1. Synthesis of *NO_2*-Bimbe, *$(NO_2)_3$*-Bimbe, *$NO_2(CF_3)_2$*-Bimbe, *$MeO(NO_2)_2$*-Bimbe and *$MeO(CF_3)_2$*-Bimbe. Reagents and conditions: (i) $POCl_3$, reflux; (ii) HNO_3/H_2SO_4, 0 °C to room temperature; (iii) 4-trifluoromethyl-benzene-1,2-diamine, MeOH, room temperature; (iv) MeI, K_2CO_3, acetone, reflux; (v) $LiAlH_4$, anhydrous THF, reflux; (vi) PCC, CH_2Cl_2; and (vii) DDQ and dioxane for *$MeO(NO_2)_2$*-Bimbe; MeOH for *$MeO(CF_3)_2$*-Bimbe; room temperature.

The five new *m*-Bimbe derivatives, *NO_2*-Bimbe, *$(NO_2)_3$*-Bimbe, *$NO_2(CF_3)_2$*-Bimbe, *$MeO(NO_2)_2$*-Bimbe and *$MeO(CF_3)_2$*-Bimbe, were synthesized according to the route shown in Scheme 1. Thus, condensation of 5-nitro-isophthalic acid with benzene-1,2-diamine afforded

NO_2-Bimbe, which was nitrated with HNO_3/H_2SO_4 to give *$(NO_2)_3$*-Bimbe [35]. Nitration of benzene-1,3-dicarbaldehyde (to give 5-nitrobenzene-1,3-dicarbaldehyde) [39] and subsequent condensation with 4-trifluoromethyl-benzene-1,2-diamine afforded *$NO_2(CF_3)_2$*-Bimbe. 5-Methoxyisophthalaldehyde was prepared starting from 5-methoxy-isophthalic acid dimethyl ester [40-42], and reacted with 4-nitro-benzene-1,2-diamine or 4-trifluoromethyl-benzene-1,2-diamine to give *$MeO(NO_2)_2$*-Bimbe and *$MeO(CF_3)_2$*-Bimbe, respectively. These compounds were fully characterized on the basis of NMR (^{1}H and ^{13}C) and ESI MS (LR and HR) data (see supporting information).

2.2 Anion recognition

In principle, the binding of the anions that are to be transported is required for a synthetic anion transporter to exert its anion transport activity [43]. Therefore, the ability of *NO_2*-Bimbe, *$(NO_2)_3$*-Bimbe, *$NO_2(CF_3)_2$*-Bimbe, *$MeO(NO_2)_2$*-Bimbe and *$MeO(CF_3)_2$*-Bimbe as receptors for chloride anions was firstly studied by means of ESI MS. ESI MS spectrometry has been widely utilized as a rapid, sensitive and effective analytical technique for the detection of non-covalent species, and the ESI MS results are assumed to reflect the complexation phenomena in solution [44]. The negative ESI MS spectra of these compounds with tetra(n-butyl)ammonium chloride (n-$Bu_4N^+ \bullet Cl^-$) are shown in Fig. 2 and S20, and indicate that they are able to form stable 1 : 1 complexes with n-$Bu_4N^+ \bullet Cl^-$ in $CH_3CN–H_2O$ (9/1, v/v). For example, in the ESI MS spectrum of *$NO_2(CF_3)_2$*-Bimbe with n-$Bu_4N^+ \bullet Cl^-$ (Fig. 2, bottom), in addition to the ion peak at *m/z* 490.40 ([*$NO_2(CF_3)_2$*-Bimbe – H]$^-$), an abundant ion peak at *m/z* 526.37 was observed. This ion peak is assigned to the 1 : 1 complex of *$NO_2(CF_3)_2$*-Bimbe with chloride anions ([*$NO_2(CF_3)_2$*-Bimbe + Cl]$^-$). The high relative abundances of the complexes suggest that these compounds exhibit high affinity toward chloride anions.

To quantitatively assess the binding ability of NO_2-Bimbe, $(NO_2)_3$-Bimbe, $NO_2(CF_3)_2$-Bimbe, $MeO(NO_2)_2$-Bimbe and $MeO(CF_3)_2$-Bimbe toward chloride anions, we measured their association constants (K_a's) with n-Bu_4N^+•Cl^- in CH_3CN–H_2O (9/1, v/v) by means of spectrophotometric titrations (Fig. S21, Tables 1 and S1). The results indicate that these compounds exhibit high binding affinity toward chloride anions. As expected, the derivatives having a 5-nitro group at the central aromatic ring tend to bind chloride anions more strongly than the corresponding analogues having no 5-substituents or a 5-methoxy group, suggesting that the addition of a 5-nitro group tends to increase the binding affinity. This improved binding ability is considered as a likely consequence of the strong electron-withdrawing properties of the 5-nitro group that increases the acidity of the –C_2H and thereby the binding affinity with anions through enhanced CH···anion interactions.

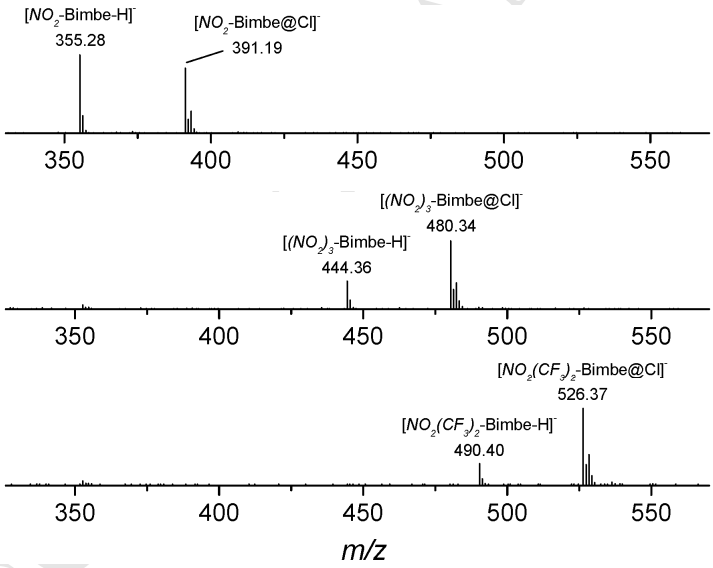


Fig. 2. Negative ESI MS spectra of 4.1 mM of NO_2-Bimbe (top), $(NO_2)_3$-Bimbe (middle) and $NO_2(CF_3)_2$-Bimbe (bottom) with n-Bu_4N^+•Cl^- (41 mM) in CH_3CN–H_2O (9/1, v/v).

The involvement of the –C_2H in the recognition of chloride anions most probably through CH···anion interactions, was confirmed by ^{1}H NMR titrations. As shown in Fig. 3 and S22, upon the addition of n-Bu_4N^+•Cl^-, the –C_2H proton experiences large complexation-induced downfield shift (CIS), a typical characteristic of hydrogen-bonding interactions [36]. Under the same conditions, the –C_2Hs in NO_2-Bimbe, $(NO_2)_3$-Bimbe and $NO_2(CF_3)_2$-Bimbe exhibit

larger downfield shifts than those in the corresponding analogues with no 5-substituents or a 5-methoxy group at the central phenyl subunits, indicative of stronger interactions with chloride anions. In addition, the -$C_{4(6)}$H protons are also downfield shifted, which may be ascribed to their intramolecular hydrogen-bonding interactions with the lone pair of the benzimidazolyl nitrogen atoms, as reported by Tiburcio *et al* [36].

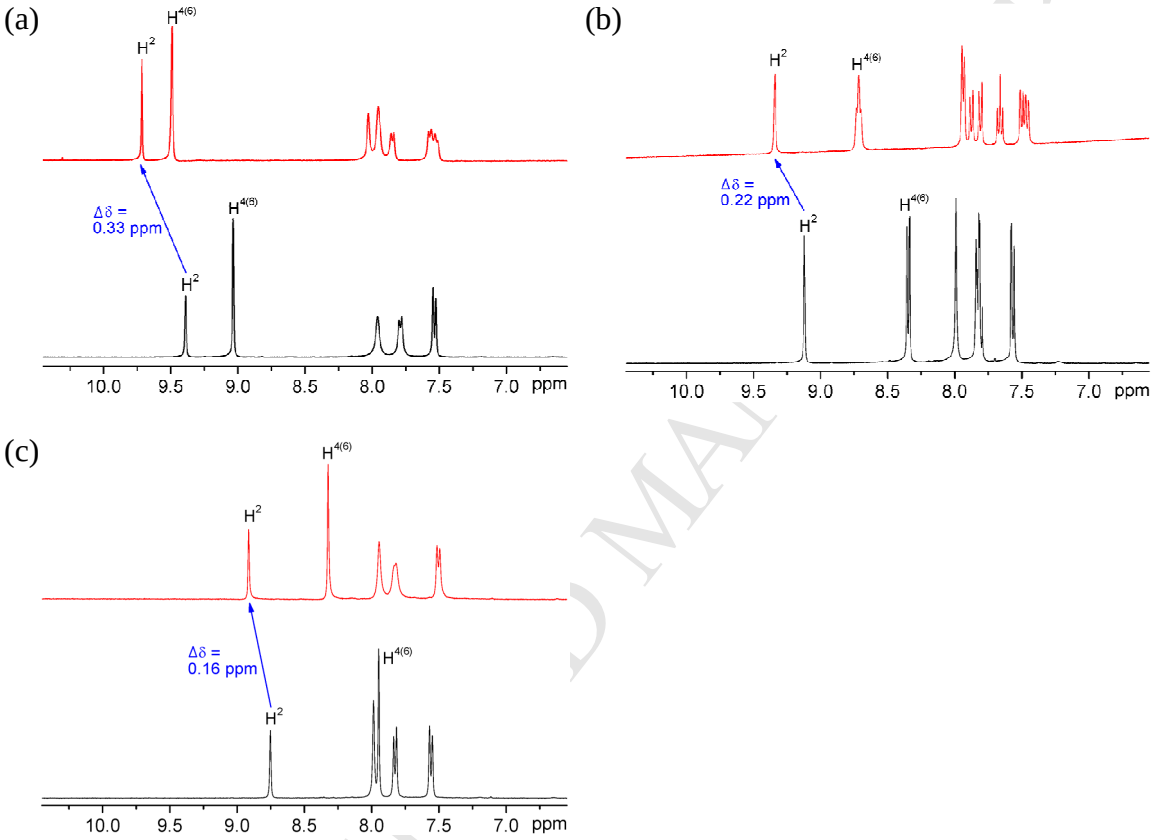


Fig. 3. ^{1}H NMR (400 MHz, DMSO-d_6) of 20 mM of (a) *$NO_2(CF_3)_2$*-Bimbe, (b) *$(CF_3)_2$*-Bimbe and (c) *$MeO(CF_3)_2$*-Bimbe before (bottom) and after (top) the addition of 30 equivalents of n-Bu_4N^+•Cl^-.

2.3 Anion transport activity

2.3.1 Anionophoric activity in liposomal models

Anionophoric activity. The ionophoric activity of *NO_2*-Bimbe, *$(NO_2)_3$*-Bimbe, *$NO_2(CF_3)_2$*-Bimbe, *$MeO(NO_2)_2$*-Bimbe and *$MeO(CF_3)_2$*-Bimbe was firstly confirmed by examining their ability to eliminate a pH differential across liposomal membranes derived from egg-yolk *L-α*-phosphatidylcholine (EYPC) [20, 45]. The data are shown in Fig. 4a and S23, and indicate

that these compounds are capable of efficiently discharging the pH differential in a concentration-dependent fashion.

To gain quantitative insights into the transport efficiency of each compound, we calculated the initial rate constants (k_{in}'s, Table S2) from each pH discharge profile and analyzed their relationship with the concentrations of each compound by using the Hill equation, $k_{in} = k_0 + k_{max}[\text{compound}]^n/([\text{compound}]^n + EC_{50}{}^n)$. This analysis gives the EC_{50} value of each compound that is the effective transporter loading when 50% of the maximum rate (k_{max}) is reached, and the Hill coefficient *n* value that defines the stoichiometry of the transport process (Fig. S24) [46]. The results, together with those of *m*-Bimbe, *(NO₂)₂*-Bimbe and *(CF₃)₂*-Bimbe for comparison, are listed in Table 1.

It is clear from Table 1 that *NO₂(CF₃)₂*-Bimbe, *(NO₂)₃*-Bimbe and *NO₂*-Bimbe exhibit highly efficient transport activity with the EC_{50} values being 2.73×10^{-3}~0.49 mol% transporter/lipid (or 0.036~6.50 μM under the assay conditions), 13~2370-fold lower than that obtained for *m*-Bimbe (EC_{50} = 6.47 mol%, or 85.6 μM). *NO₂*-Bimbe, *NO₂(CF₃)₂*-Bimbe and *(NO₂)₃*-Bimbe exhibit 13-, 26- and 3-fold higher activity than their corresponding analogues without a 5-nitro group, that is, *m*-Bimbe, *(CF₃)₂*-Bimbe and *(NO₂)₂*-Bimbe, respectively. Replacement of the 5-nitro group in *NO₂(CF₃)₂*-Bimbe and *(NO₂)₃*-Bimbe with a methoxy group (to give *MeO(CF₃)₂*-Bimbe and *MeO(NO₂)₂*-Bimbe, respectively) led to 40- and 120-fold decrease in the activity, respectively. These results unambiguously reveal the crucial role of an electron-withdrawing 5-substituent in enhancing the transport efficiency. It is remarkable that *NO₂(CF₃)₂*-Bimbe and *(NO₂)₃*-Bimbe have very low EC_{50} values of 3.76×10^{-3} mol% (or 50 nM) and 2.73×10^{-3} mol% (or 36 nM), respectively, suggesting that they are very active transporters. More significantly, both *NO₂(CF₃)₂*-Bimbe and *(NO₂)₃*-Bimbe can still exhibit detectable pH discharge activity at the loadings as low as 4.48×10^{-4} mol% transporter/lipid (or

5.9 nM) (Fig. 4a and S23). Calcein leakage assay indicates that the activity is authentic, not due to the disruption of the liposomal membranes by these compounds (Fig. S25) [47].

The high ionophoric activity of these compounds is considered as a likely consequence of the enhanced binding affinity by the 5-nitro group. Because lipophilicity, widely measured by the logarithm of n-octanol/water partition coefficient *P* (log *P*), is one of the major factors that regulate the activity of an anion transporter [4, 12], we calculated the log *P* value (*c*log*P*) of each compound (Table 1). The results indicate that the 5-nitro group does not significantly change the *c*log*P* values of the related compound set (for example, *m*-Bimbe and NO_2-Bimbe), which rules out the possibility that the high activity is due to the alteration in the lipophilicity [4, 12]. The finding that the EC_{50} values tend to decrease with the increase in the binding affinity (Table 1), suggests that the transport activity may be largely regulated by the binding ability of these compounds with chloride anions.

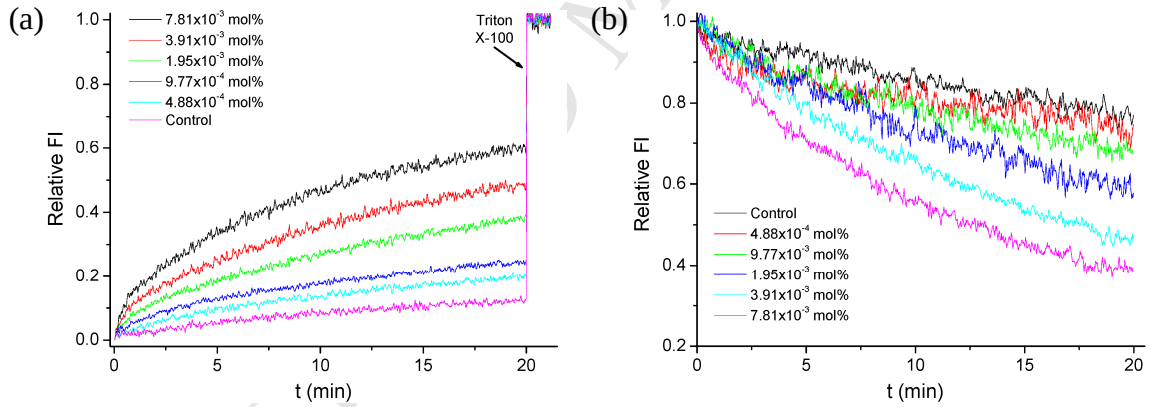


Fig. 4. (a) Discharge of a pH gradient across EYPC-based liposomal membranes by $NO_2(CF_3)_2$-Bimbe of varying concentrations. Intravesicular conditions: 0.1 mM pyranine in 25 mM HEPES (50 mM NaCl, pH 7.0); Extravesicular conditions: 25 mM HEPES (50 mM NaCl, pH 8.0). λ_{Ex} 460 nm/λ_{Em} 510 nm. (b) Chloride influx into EYPC vesicles promoted by $NO_2(CF_3)_2$-Bimbe of varying concentrations. Intravesicular conditions: 1 mM lucigenin in 25 mM phosphate buffer (225 mM $NaNO_3$, pH 7.0); Extravesicular conditions: 25 mM NaCl in 25 mM phosphate buffer (225 mM $NaNO_3$, pH 7.0). λ_{Ex} 455 nm/λ_{Em} 506 nm. All the experiments were performed in triplicate, and the mean values were taken.

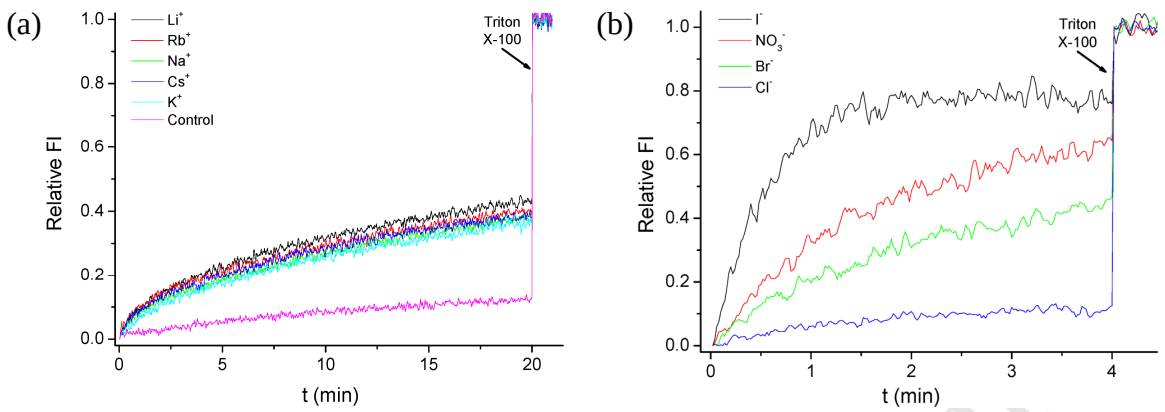


Fig. 5. Discharge of a pH gradient by $NO_2(CF_3)_2$-Bimbe (1.95×10^{-3} mol%) across EYPC-based liposomal membranes. (a) Measuring conditions for internal vesicles: 0.1 mM pyranine in 25 mM HEPES (50 mM MCl, pH 7.0) and external vesicles: 25 mM HEPES (50 mM MCl, pH 8.0) (M = Li, Na, K, Rb or Cs). The experiment that was conducted in NaCl media was used as a control. (b) Measuring conditions for internal vesicles: 0.1 mM pyranine in 25 mM HEPES (50 mM NaX, pH 7.0) and external vesicles: 25 mM HEPES (50 mM NaX, pH 8.0) (X = NO_3, Cl, Br or I). Each profile was obtained by subtracting the background and thereby represents the real increment in the presence of $NO_2(CF_3)_2$-Bimbe.

Table 1. Association constants (K_a's) and kinetic parameters for pH discharge [a]

Compound	K_a (M^{-1}) [b]	*clogP* [c]	Kinetic parameters [d]			
			n	EC_{50} (mol%)	EC_{50} (μM) [e]	RA [f]
m-Bimbe	$(2.48\pm0.82)\times10^3$	4.60	1.73±1.33	6.47	85.8	1
NO_2-Bimbe	$(3.94\pm1.19)\times10^3$	4.54	1.25±0.47	0.49	6.50	13
$(NO_2)_2$-Bimbe	$(2.22\pm0.79)\times10^3$	4.48	1.38±0.36	8.19×10^{-3}	0.11	790
$(NO_2)_3$-Bimbe	$(8.18\pm0.96)\times10^3$	4.42	1.19±0.18	2.73×10^{-3}	0.036	2370
$MeO(NO_2)_2$-Bimbe	$(1.56\pm0.46)\times10^3$	4.32	0.99±0.18	0.32	4.24	20
$(CF_3)_2$-Bimbe	$(4.52\pm0.82)\times10^3$	6.35	1.11±0.26	9.59×10^{-2}	1.27	67
$NO_2(CF_3)_2$-Bimbe	$(7.53\pm0.91)\times10^3$	6.29	1.31±0.06	3.76×10^{-3}	0.05	1721
$MeO(CF_3)_2$-Bimbe	$(3.53\pm0.88)\times10^3$	6.19	0.85±0.04	0.15	1.99	43

[a] The data for *m*-Bimbe, $(NO_2)_2$-Bimbe and $(CF_3)_2$-Bimbe have been reported previously [35].

[b] Measured by means of spectrophotometric titrations in CH_3CN-H_2O (9/1, v/v).

[c] Calculated using MarvinSketch (Version 6.1.0, Weighted Model, ChemAxon, MA).

[d] See Fig. 4a and S23 for the detailed assay conditions.

[e] The EC_{50} values in μM were calculated by multiplying the concentration of EYPC (1.33 mM) by the EC_{50} in mol%.

[f] RA denotes the transport efficiency of each compound relative to *m*-Bimbe.

Probable mechanism of action The anion selectivity and the probable mechanism of action of *NO_2*-Bimbe, *$(NO_2)_3$*-Bimbe and *$NO_2(CF_3)_2$*-Bimbe were investigated firstly by measuring the pH discharge activity in the presence of different alkali metal ions or anions. The finding that the activity is essentially independent of the alkali metal ions (Fig. 5a and S26), whereas varies with the anions in the order of $I^- > NO_3^- > Br^- > Cl^-$ (Fig. 5b and S27), suggests that the pH gradient decay is a consequence of OH^-/X^- antiport and/or H^+/X^- symport [48]. The anion-selective transport activity of *NO_2*-Bimbe, *$(NO_2)_3$*-Bimbe and *$NO_2(CF_3)_2$*-Bimbe was confirmed by measuring their ability to mediate the influx of chloride anions into the EYPC-derived liposomes, using a conventional fluorescence method based on a chloride-sensitive fluorescent dye, lucigenin [49]. As shown in Fig. 4b and S28, these compounds exhibit powerful chloride influx activity. Interestingly, the chloride influx was found to be significantly suppressed when the vesicles entrapped with highly hydrophilic sulfate were used (Fig. S29), suggesting that anion exchange is a predominant step in the permeation process [49]. However, a minor level of H^+/Cl^- symport may be also involved, as the chloride influx was slightly affected when sulfate was present in both internal and external vesicles, under the condition of which Cl^-/SO_4^{2-} antiport is not favored (Fig. S30) [34, 35].

In addition, the finding that the pH discharge activity of *NO_2*-Bimbe, *$(NO_2)_3$*-Bimbe and *$NO_2(CF_3)_2$*-Bimbe was significantly reduced in liposomes derived from POPC with 30% cholesterol (Fig. S31), supports a mobile carrier mechanism. This is consistent with the Hill coefficient *n* values being around 1.

The above-mentioned observations suggest that *NO_2*-Bimbe, *$(NO_2)_3$*-Bimbe and *$NO_2(CF_3)_2$*-Bimbe function as anion-selective, highly effective carriers through a process of anion exchange with a minor level of proton/anion symport.

2.3.2 *In vitro* anionophoric activity

The potent anionophoric activity of these compounds in liposomal models inspired us to study their anionophoric activity in cells. For comparative purpose, we chose four compounds of this set, that is, the parent compound *m*-Bimbe, $NO_2(CF_3)_2$-Bimbe having a 5-nitro group at the central phenyl subunit and trifluoromethyl groups at the benzimidazoloyl subunits, and the corresponding analogues $(CF_3)_2$-Bimbe and $MeO(CF_3)_2$-Bimbe having no 5-substituents or a 5-methoxy group at the central phenyl subunits. Firstly, we utilized the MQAE [*N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide] assay to measure the chloride entry into cells in the presence of those four compounds [5, 6, 9]. MQAE is a cell-permeable, chloride selective dye and its fluorescence is quenched by the anion. Thus, the fluorescence quenching of MQAE upon the treatment of cells with *m*-Bimbe, $(CF_3)_2$-Bimbe, $MeO(CF_3)_2$-Bimbe and $NO_2(CF_3)_2$-Bimbe can be the direct evidence that these compounds are involved in the transmembrane transport of chloride anions into the cells [5, 6, 9]. As shown in Fig. 6, post incubation of either HeLa cervical cancer cells or MCF-7 breast cancer cells with these compounds led to significant quenching of the MQAE fluorescence, which indicates the influx of chloride anions in the intracellular matrix facilitated by these compounds. Interestingly, this *in vitro* chloride transport activity of these compounds that is expressed as the fluorescence quenching of MQAE parallels their anion transport efficiency in liposomal models. It should be noted that at this stage we have no evidences for whether the compounds themselves or the biological chloride channels/transporters activated by the compounds mediate the influx of chloride anions into the HeLa or MCF-7 cells.

The *in vitro* anionophoric activity of *m*-Bimbe, $(CF_3)_2$-Bimbe, $MeO(CF_3)_2$-Bimbe and $NO_2(CF_3)_2$-Bimbe in HeLa cell lines, was further studied using vital staining with acridine orange (AO), a cell-permeable dye [3,4]. When protonated and accumulated in acidic compartments such as lysosomes, AO exhibits a characteristic orange fluorescence emission,

whereas it emits green fluorescence when the acidic compartments are basified. As shown in Fig. 7 and S32, when HeLa cells were stained with AO, granular orange fluorescence was observed in the cytoplasm (Fig. 7a), suggesting that the orange fluorescence is due to acidic organelles. Treatment of the HeLa cells with these compounds led to disappearance of most of the orange emission for *m*-Bimbe and *MeO*$(CF_3)_2$-Bimbe (Fig. 7b and 7c), and complete disappearance of the orange emission for $(CF_3)_2$-Bimbe and $NO_2(CF_3)_2$-Bimbe (Fig. 7d and 7e), respectively. This result suggests that these compounds, in particular $NO_2(CF_3)_2$-Bimbe and $(CF_3)_2$-Bimbe are able to efficiently basify the acidic organelles. Notably, the basifying ability in the order of $NO_2(CF_3)_2$-Bimbe > $(CF_3)_2$-Bimbe > *MeO*$(CF_3)_2$-Bimbe > *m*-Bimbe, parallels their ionophoric activity in liposomal models (Table 1). The proton/chloride symport observed in the liposomal models, is thought to be responsible for the increase of the internal pH although other mechanisms (e.g., Cl^-/HCO_3^-) may also be involved [3, 4].

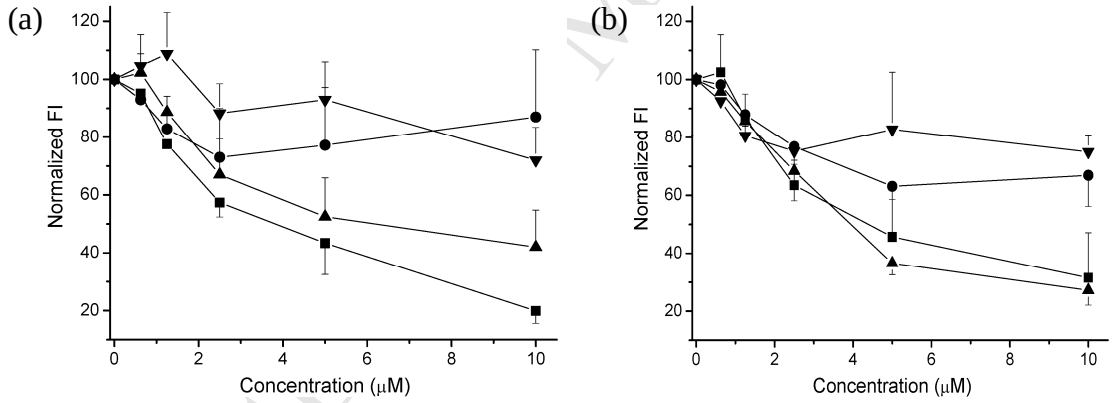


Fig. 6. Normalized fluorescence intensity of MQAE in (a) HeLa and (b) MCF-7 cells incubated with MQAE (5 mM) for 3.5 h followed by the treatment with *m*-Bimbe (▼), $(CF_3)_2$-Bimbe (▲), *MeO*$(CF_3)_2$-Bimbe (●) and $NO_2(CF_3)_2$-Bimbe (■) of varying concentrations for 2 h. Fluorescence intensity was recorded by the plate reader at λ_{em} = 460 nm (λ_{ex} = 350 nm), and normalized with respect to the fluorescence intensity of untreated cells. Each data point represents the mean intensity of three independent experiments.

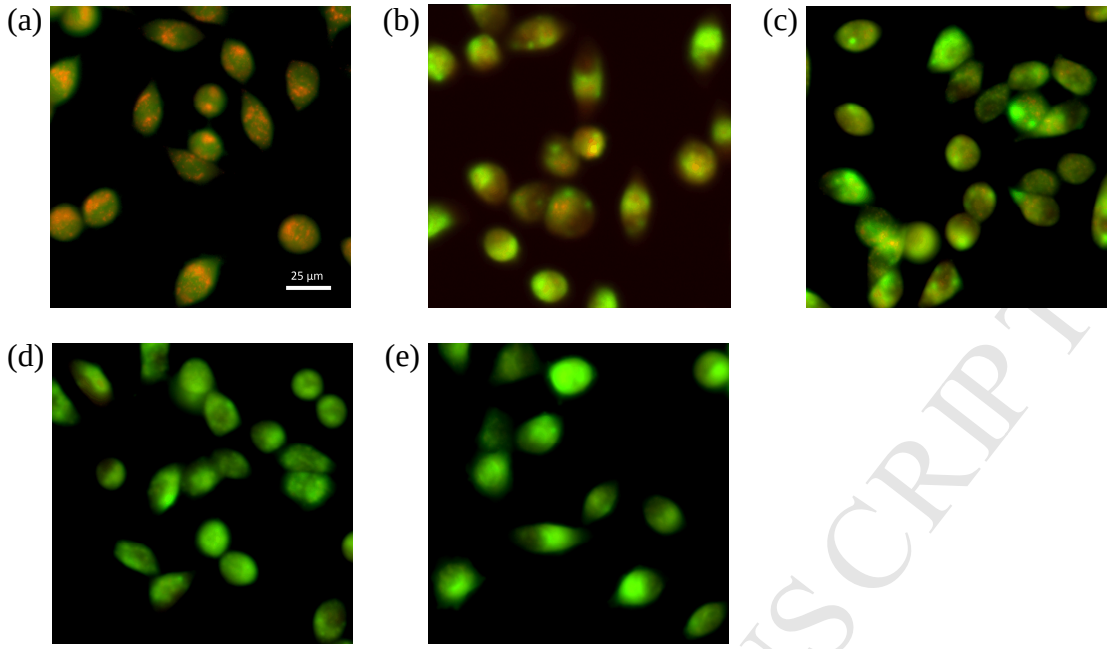


Fig. 7. Acridine orange staining of HeLa cell lines. (a) Untreated cells (control); (b)-(e) Cells treated with 10 μM of *m*-Bimbe, *MeO*$(CF_3)_2$-Bimbe, $(CF_3)_2$-Bimbe and $NO_2(CF_3)_2$-Bimbe for 3 h, respectively.

2.4 Anti-proliferative activity

2.4.1 *Cytotoxicity*

As these compounds are able to mediate the transport of chloride anions into live cells and previous studies have shown that stimulated influx of chloride anions into cells induces cell death [8, 9, 50], we tested the *in vitro* cytotoxicity of *m*-Bimbe and all the derivatives described in Fig. 1 using a conventional MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Initially single-dose screening (50 μM) was carried out to evaluate the inhibitory activity of each compound toward four solid tumor cell lines, including HeLa, A549 lung adenocarcinoma, MCF-7 and HepG2 human liver cancer cell lines. The cell viability was reported as a percentage of control cells (Fig. 8 and Table S3). The IC_{50} values of those active compounds in the single-dose cytotoxicity tests at 50 μM, are listed in Fig. S33-S42, Tables 2 and S4-S5. For comparison, the IC_{50} values of all the active compounds in the single-dose screening toward LO2 human normal liver cells were also measured (Tables 2 and S6). Here the IC_{50} values represent the concentration of each compound resulting in 50%

inhibition in cell growth. Doxorubicin was used as a positive control.

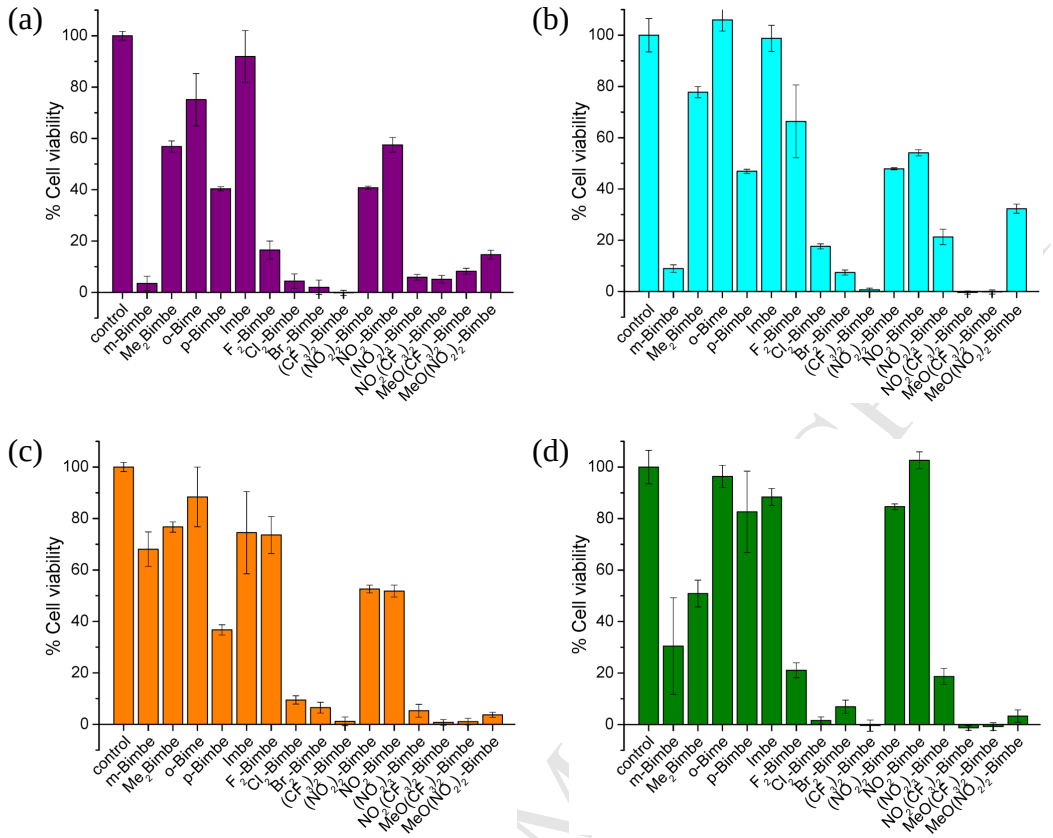


Fig. 8. Cell viability of *m*-Bimbe and its derivatives (50 μM) toward (a) HeLa, (b) A549, (c) MCF-7 and (d) HepG2 cancer cell lines.

Table 2. Cytotoxicity (IC_{50}) of *m*-Bimbe and its derivatives towards cancerous and normal cells

Compound	IC_{50} (μM)				
	HeLa	A549	MCF-7	HepG2	LO2
m-Bimbe	8.5±0.7	38.5±4.0	> 100	20.6±7.0	19.9±1.0
F_2-Bimbe	11.6±0.5	> 100	> 100	17.3±4.6	20.8±3.7
Cl_2-Bimbe	10.7±1.5	23.7±3.7	25.9±2.8	9.1±0.2	13.7±1.5
Br_2-Bimbe	21.1±0.1	21.9±0.9	9.7±4.2	12.1±2.1	17.8±1.2
$(CF_3)_2$-Bimbe	8.0±1.5	8.9±0.6	12.8±0.9	6.6±0.6	10.9±0.6
$(NO_2)_3$-Bimbe	1.2±0.1	13.6±4.2	1.2±0.3	11.1±1.3	44.9±4.5
$NO_2(CF_3)_2$-Bimbe	1.9±0.3	3.9±0.7	9.7±0.5	4.1±0.8	3.7±0.2
$MeO(CF_3)_2$-Bimbe	8.4±1.2	8.1±1.9	11.9±0.3	10.7±0.3	10.1±1.4
$MeO(NO_2)_2$-Bimbe	1.0±0.2	18.5±6.1	17.1±0.6	8.9±1.0	26.3±2.0
Doxorubicin	0.5±0.1	0.6±0.1	0.8±0.1	2.1±0.2	0.11±0.03

It is evident that most of the compounds exhibit potent cytotoxicity toward the four cells, and some of them are even comparable with doxorubicin. Though little selective cytotoxicity for the cancer cells over the LO2 normal cells was observed, analysis of the cytotoxicity may afford some preliminary structure-activity relationships, which may provide some useful guidance for future rational design of *m*-Bimbe-based anion transporters.

Firstly, *m*-Bimbe shows much higher inhibitory activity than *o*-Bime, *p*-Bimbe, Imbe and Me_2Bimbe. This result suggests that attaching two benzimidazolyl groups in the *meta*-position of a phenyl group and keeping the benzimidazolyl NH motifs are required not only for anion transport activity [35], but also for cytotoxicity. *o*-Bime, *p*-Bimbe, Imbe and Me_2Bimbe that are inactive as anion transporters [35], are essentially non-cytotoxic. This finding implies that a compound without anion transport activity exhibits a very low level of cytotoxicity (if there is any).

Secondly, the effect of the substituents at the benzimidazolyl subunits on the inhibitory activity varies with the substituents. For example, compared with *m*-Bimbe, F_2-Bimbe, Cl_2-Bimbe, Br_2-Bimbe and $(CF_3)_2$-Bimbe exhibit better or comparable activity. However, $(NO_2)_2$-Bimbe almost loses its cytotoxic effect. These results suggest that adding electron-withdrawing substituents onto the benzimidazolyl subunits does not necessarily increase the cytotoxicity, though such substituents are favorable to the anion transport activity [35]. Among all the substituents, trifluoromethyl group is the most favorable one for the cytotoxicity.

Thirdly, the *m*-Bimbe derivatives having a 5-nitro group at the central phenyl subunit, such as $NO_2(CF_3)_2$-Bimbe and $(NO_2)_3$-Bimbe are much more cytotoxic than their corresponding analogues without a 5-nitro group, that is $(CF_3)_2$-Bimbe and $(NO_2)_2$-Bimbe, respectively. However, it is unexpected that $MeO(CF_3)_2$-Bimbe and $MeO(NO_2)_2$-Bimbe with much lower anion transport activity, also exhibit potent cytotoxicity. This result implies that other

mechanisms besides anion transport may be also responsible for the cytotoxic effects [51].

Overall, the 5-nitrated *m*-Bimbe derivative having trifluoromethyl groups at the benzimidazoloyl subunits, that is, $NO_2(CF_3)_2$-Bimbe exhibits the most favorable cytotoxicity (and anion transport activity), and therefore was used for further mechanistic study.

2.4.2 *Effect of chloride anions on cytotoxic effects*

It is known that live cells involve a lot of complex ion homeostasis that are responsible for antitumor activity [5, 6, 8]. As shown in Fig. 6-7 and Table 2, the entry of chloride anions into live cells can be facilitated in the presence of $NO_2(CF_3)_2$-Bimbe, $(CF_3)_2$-Bimbe, $MeO(CF_3)_2$-Bimbe or *m*-Bimbe, and their *in vitro* anionophoric activity observed in the MQAE assay and AO staining, parallels their cytotoxicity. These results imply that chloride transport across cellular membranes may be responsible for the cytotoxic effects. To gain support for this, we measured the cytotoxicity of $NO_2(CF_3)_2$-Bimbe toward HeLa and MCF-7 cell lines in the presence and the absence of chloride anions. As shown in Fig. 9, $NO_2(CF_3)_2$-Bimbe was found to be more cytotoxic toward both HeLa and MCF-7 cell lines in the presence of chloride anions than in the absence of chloride anions. This result strongly suggests that chloride transport plays a crucial role in the cytotoxic effects [5, 6, 8].

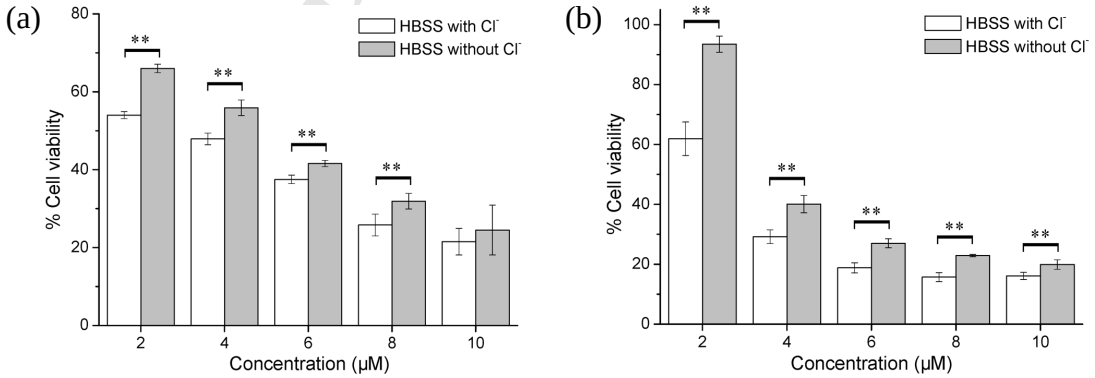


Fig. 9. Cell viability of (a) HeLa and (b) MCF-7 cells in the Cl^--containing and Cl^--free extracellular media (HBSS buffer) upon the dose-dependent treatment of $NO_2(CF_3)_2$-Bimbe (2−10 μM) for 12 h (mean ± s.d., n = 4, $^{**}P < 0.01$). The differences in mean intensities are statistically significant ($P < 0.01$) according to one-way analysis of variance (ANOVA).

2.4.3 *Probable mechanism of action for cell death*

Literature reports have shown that dysregulation of ion homeostasis, in particular *via* chloride influx, can induce cell shrinkage and lead to apoptosis [6, 9, 50]. To gain insight into how these compounds induce cell death, we used Hoechst 33342 staining to investigate the effect of $NO_2(CF_3)_2$-Bimbe on HeLa cell lines. Hoechst 33342 staining is able to differentiate apoptosis from other cell death mechanisms through morphological observation [4, 6]. As shown in Fig. 10, compared with the untreated cells, the cells treated with $NO_2(CF_3)_2$-Bimbe exhibit stronger blue fluorescence, indicative of chromatin condensation, fragmentation and apoptotic bodies formation. The formation of "bean"-shaped nuclei was also observed. These are typical features for cell death *via* an apoptotic process.

To further confirm the apoptotic mechanism of action, we used a JC-1 probe to observe the loss of mitochondrial membrane potential in HeLa cells, a hallmark of apoptosis [6, 52]. JC-1 probe is a cell-permeable dye the fluorescence of which is sensitive to mitochondrial membrane potential. As shown in Fig. 11, after the HeLa cells were treated with $NO_2(CF_3)_2$-Bimbe of varying concentrations and then with JC-1, the pixel intensity ratio of the red fluorescence of JC-1 to the green fluorescence decreases significantly. This is indicative of the decrease of the mitochondrial membrane potential, and is one of the characteristics for apoptotic cell death in an early stage.

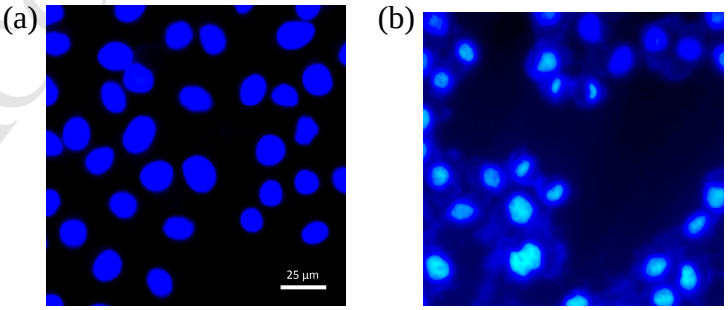


Fig. 10. Hoechst 33342 staining of HeLa cell lines. (a) Untreated (control) cells; (b) cells treated with $NO_2(CF_3)_2$-Bimbe (2.5 µM).

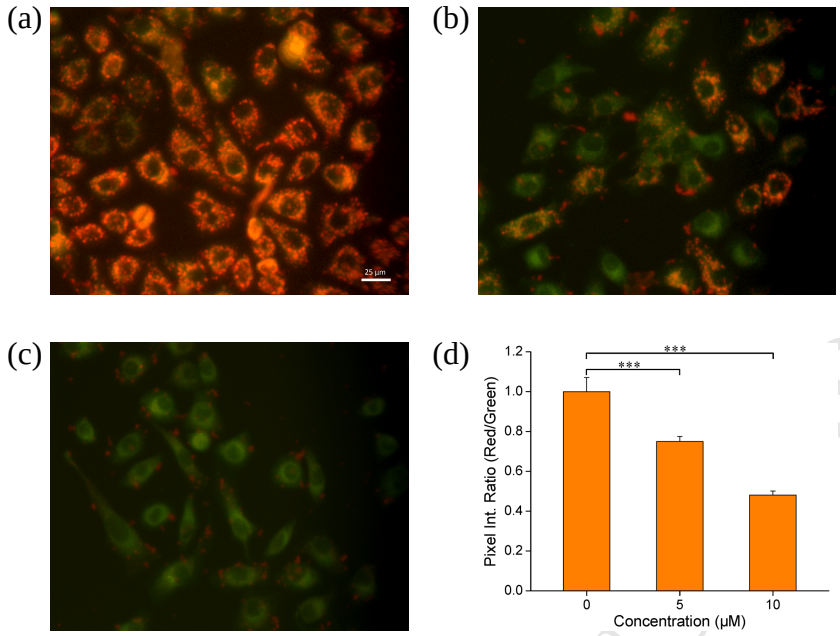


Fig. 11. JC-1 staining of HeLa cell lines. (a) Untreated cells; (b)-(c) cells treated with $NO_2(CF_3)_2$-Bimbe at the concentrations of 5 μM and 10 μM, respectively; (d) Pixel ratio (red/green) for different concentrations of $NO_2(CF_3)_2$-Bimbe, analyzed by ImageJ. The error bars mean that the ratio of pixel intensities was calculated from nine different horizons (mean ± s.d., n = 9, ***$P < 0.001$).

Taken together, the results obtained from Hoechst 33342 and JC-1 staining provide evidences for the apoptosis-inducing activity of $NO_2(CF_3)_2$-Bimbe.

3. Conclusion

In conclusion, we have synthesized a series of *m*-Bimbe derivatives and investigated in details their anion transport activity, anti-proliferative activity and probable mechanism of action. Firstly, we have demonstrated that adding a strong electron-withdrawing nitro group at the 5-position of the central phenyl subunits of *m*-Bimbe and its derivatives can greatly improve the anion transport efficiency. This effect is considered due to the enhanced CH···anion interactions, as evidenced from ESI MS, spectrophotometric and ^{1}H NMR titrations. *In vitro* anionophoric activity investigated by MQAE assay and AO vital staining indicates that these compounds can disturb the homeostasis of chloride anions, modify the intracellular pH and induce the basification of acidic organelles. We have measured the cytotoxicity of this series

of *m*-Bimbe derivatives toward four solid tumor cell lines, using the conventional MTT assays. The results indicate that most of the *m*-Bimbe derivatives exhibit potent cytotoxicity, and the 5-nitrated derivative bearing trifluoromethyl groups at the benzimidazoloyl subunits is the most active with the IC_{50} value in the low micromolar range. The findings that the entry of chloride anions into live cells, evaluated by use of MQAE assay, parallels the anion transport efficiency and the cytotoxicity, and these compounds exhibit higher cytotoxicity in the presence of chloride anions, suggest that the transport of chloride anions across the cellular membranes plays a critical role in the cytotoxic effects. Preliminary mechanistic study by using Hoechst 33342 and JC-1 staining suggests that these compounds induce cell death most probably *via* an apoptotic process. Hence, an *m*-Bimbe derivative that has both high anion transport efficiency and biological activity (for example, disordering ion homeostasis) may be developed as a potential apoptotic agent for tumor treatment. Further efforts are aimed at clarifying the detailed mechanism of action of these compounds and creating structurally optimized benzimidazole-based anion transporters with exceptionally high activity. The outcomes will be reported in due course.

Supporting information

Experimental procedures and data for the synthesis, structural characterization, anion binding affinity, transmembrane anion transport activity and biological activity of each compound.

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Research Highlights

► A series of 1,3-bis(benzimidazol-2-yl)benzene derivatives were synthesized.

► The anionophoric activity was greatly improved by adding electron-withdrawing groups.

► These derivatives exhibit potent anionophoric activity in liposomal models and cells.

► Most of these derivatives exhibit potent cytotoxicity toward the tested cancer cells.

► These derivatives induce cell death most probably *via* an apoptotic process.