Enhanced cytotoxicity of benzimidazole carbamate derivatives and solubilisation by encapsulation in cucurbit[*n*]uril†

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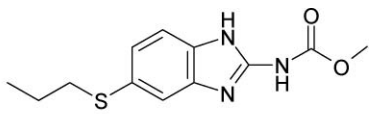
The albendazole derivatives (2-methoxyethyl) 5-propylthio-1*H*-benzimidazole-2-yl carbamate (MEABZ), N1-(2-methoxyethoxycarbonyl)-2-amino-5-propylthiobenzimidazole and N1-(2-methoxyethoxycarbonyl)-2-amino-6-propylthiobenzimidazole (MEABZ isomers A and B) and (2-hydroxyethyl) 5-propylthio-1*H*-benzimidazole-2-yl carbamate (HEABZ) have been synthesised. The cytotoxicity of these compounds was evaluated against a human colorectal cancer cell line (HT-29) and a human prostate cancer cell line (PC-3). The results demonstrate MEABZ, a new benzimidazole, is up to ten times more cytotoxic than the parent drug albendazole, whereas the MEABZ isomers A and B and HEABZ show no activity. A comparison of the cytotoxicity of these compounds, relative to ABZ, provides structure–activity data for this important class of anticancer agents. The aqueous solubilities of MEABZ encapsulated in Q[*n*] have been determined by ^{1}H NMR spectroscopy. The aqueous solubility of MEABZ at a physiologically relevant pH increased by 1200-fold by encapsulation in Q[8], from 8 μM to 9.4 mM, while Q[6,7] encapsulation substantially increased the solubility to more than 2 mM. Encapsulation in Q[7] and Q[8] induced significant upfield shifts for the MEABZ propyl and benzimidazole resonances. The upfield shifts indicate that the propyl and benzimidazole protons are located within the Q[7] and Q[8] cavity upon encapsulation. By contrast, encapsulation in Q[6] induced large upfield shifts for the ^{1}H resonances from the carbamate functional group, indicating that MEABZ associates with Q[6] at its portals, with only the carbamate group binding within the cavity.

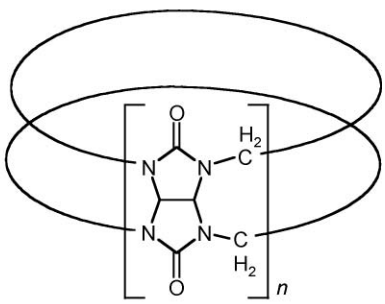
Introduction

Albendazole (ABZ), methyl 5-propylthio-1*H*-benzimidazole-2-yl carbamate (Fig. 1), is a highly efficacious well known antiparasitic drug, with minor toxicities.[1–3] More recently, ABZ has been shown to exhibit significant activity against a range of tumours, including hepatocellular,[4] colorectal,[5] ovarian cancers[6] and paclitaxel resistant leukaemic cells.[7] The drug has also been shown to inhibit vascular endothelial growth factor and thus suppress angiogenesis and malignant ascites formation.[8]

While ABZ has huge potential in the treatment of cancer, its clinical application as an anticancer agent has been limited by its aqueous solubility. In our previous study,[10] the aqueous solubility of ABZ at pH 6.6 was found to be 3 μM, consistent with an earlier report,[9] and 17 μM at pH 3.0. We also demonstrated that encapsulation of ABZ in cucurbit[*n*]uril significantly increased the aqueous solubility of ABZ.[10] Cucurbit[*n*]uril, Q[*n*], (see Fig. 1) is a family of macrocyclic molecules made from the condensation of glycoluril and formaldehyde with hydrophobic cavities and electronegative carbonyl rimmed portals.[11] Q[*n*] can be isolated in a range of cavity sizes, with portal dimensions ranging from 2.4 to 11.0 Å for Q[5] to Q[10].[12–15] Inclusion complexes can be formed with a large variety of drug molecules.[16–18] For ABZ, encapsulation



(A)



(B)

Fig. 1 (A) albendazole (ABZ) and (B) cucurbit[*n*]uril.

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† Electronic supplementary information (ESI) available: ^{1}H NMR spectrum of ABZ-amine; ^{1}H NMR spectrum of HEABZ; Determination of the pK_a of free and Q[7]- and Q[8]-bound MEABZ; An example of the product distribution of HEABZ and ABZ-amine that occurs in the synthesis of HEABZ; Figure showing the solvolysis reaction of the MEABZ isomer B with methanol to slowly form ABZ-amine. See DOI: 10.1039/c003732j

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in Q[7] and Q[8] was found to significantly increase the aqueous solubility of the drug, with the Q[7,8] cavity accommodating the benzimidazole moiety of ABZ, with the more polar carbamate 'tail' projecting out of the portal.[10] Such a binding mode would be stabilized by hydrophobic forces and van der Waals interactions within the cavity and dipole–ion (assuming a protonated ABZ structure) and hydrogen bonding at the portals, thereby dramatically increasing the aqueous solubility by 2000-fold from μM to mM concentrations.[10]

Another approach to increasing the aqueous solubility of ABZ is through the formation of derivatives of the parent compound.[19] As ABZ binds cucurbit[*n*]uril with the benzimidazole moiety in the cavity and the carbamate group projecting out of the portal, it was proposed to modify the methoxy moiety of the carbamate to form ABZ derivatives that were more water soluble, but still capable of strong binding to Q[7,8]. Consequently, it was hoped that through encapsulation in cucurbit[*n*]uril in combination with a more soluble ABZ analogue, a highly active water soluble ABZ-type anticancer formulation could be obtained. The synthesis, Q[*n*] binding and cytotoxicity of four ABZ derivatives were explored. One compound, (2-methoxyethyl) 5-propylthio-1*H*-benzimidazole-2-yl carbamate (MEABZ), was found to be slightly more water soluble than ABZ, both in the free and Q[7,8]-bound forms, and significantly more cytotoxic than ABZ in a range of cancer cell lines.

Experimental

Materials

ABZ was purchased from the Sigma Chemical Company, while D_2O was obtained from Cambridge Isotope Laboratories. Q[6], Q[7], Q[8] were synthesized as previously described.[20,21] All solvents were used as provided and aqueous solutions were made using Milli-Q water, coming from a Millipore four-stage water purification unit.

NMR spectroscopy

NMR spectra were recorded on a Varian Unity*plus-400* spectrometer operating at 400 MHz for the ^{1}H nuclei. One-dimensional ^{1}H NMR spectra were recorded over a spectral width of 5000 Hz using 256 transients. All spectra were referenced to DSS (0 ppm) at 25 °C, using the residual HDO resonance at 4.78 ppm as an internal reference signal.

MEABZ aqueous solubility

An excess of the drug was added to 150 mL of water at either pH 3.5 or 6.6, the suspension was sonicated for 90 min and then left standing overnight at 20 °C. The suspension was then filtered through a 0.5 μm PETE syringe filter, lyophilized and then re-dissolved in 50 mL of methanol. The concentration of the drug was determined from the absorption at 295.7 nm against a standard curve.

Cucurbit[*n*]uril titrations of MEABZ

Q[7] was dissolved in D_2O to a specific concentration and then mixed with known weights of the MEABZ, whereas, Q[6], Q[8] which are sparingly soluble in water, were added to MEABZ and D_2O in solid form. All samples were shaken using a vortex shaker, sonicated and then left standing overnight before analysis by NMR spectroscopy. MEABZ concentrations in solution were measured by comparing the integrals of the MEABZ ^{1}H resonances with those of known concentrations of ethanol in D_2O, typically 3 mM. The results were corrected for the different spin–lattice (T_1) relaxation times between the ethanol and MEABZ protons. T_1 times were determined using the standard 180°-τ-90° pulse sequence. The concentration ratio of MEABZ to cucurbituril was determined by comparing the ^{1}H resonance integrals of MEABZ with those of the Q[*n*].

Standard ethanol solution

To 3.5 mg (0.0287 mmol) of benzoic acid dissolved in 5.00 mL D_2O, 1.59 mg (0.015 mmol) of sodium carbonate was added, giving a 5.73 mM sodium benzoate solution. Absolute ethanol (21 μL, density = 0.789 g mL^{-1}) was dissolved in 2.00 mL D_2O. 10 μL of the ethanol solution was added to 0.60 mL of the sodium benzoate solution. The ethanol concentration in solution was measured by comparing the integrals of the ethanol proton peaks with the known concentration of sodium benzoate.

Determination of pK_a for MEABZ

A typical preparation involved adding an excess of MEABZ to 1.0 mL of water at ~ pH 3.5, the suspension was sonicated for 90 min and then left standing overnight at 20 °C. The suspension was then filtered through a 0.5 μm PETE syringe filter. To 50 μL of the solution, exactly 5.0 mL of water (pH 2.0) was added, the pH was obtained with 5.0 M HCl. The temperature of the solution was carefully controlled at 25 °C during the titration. The solution was titrated with 0.1 M KOH to pH 6.5. The pH was measured using a calibrated pH meter and UV spectra were collected after each pH adjustment. The UV spectrum was scanned from 200–400 nm. The pK_a of each sample was taken from the mid-point of the inflection of the pH *versus* UV reading titration curve.

Preparation of MEABZ encapsulated in Q[*n*] (MEABZ@Q[*n*]) for *in vitro* testing

A typical preparation involved mixing solid MEABZ (3.1 mg, 10 μmol) and solid Q[7] (14.3 mg, 10 μmol) in H_2O (pH 2.0, 1.0 mL). Using a vortex shaker the mixture was homogenized, sonicated for 2 h and then left to stand overnight at 20 °C. Filtration through a 0.5 μm PETE syringe filter gave clear solutions of the MEABZ@Q[7] complex. The solid product was obtained by lyophilization. The mole ratio of MEABZ to Q[7] was determined by ^{1}H NMR. The exact mole ratio for each preparation was determined before use. Samples dissolved in D_2O were compared using peak integrals against additions of 10 μL of standardised ethanol (185 mM) in D_2O. The MEABZ samples for *in vitro* cytotoxicity were prepared in saline solutions in accordance with the determined MEABZ@Q[7] ratios of the above lyophilized preparations.

Cell proliferation assay of MEABZ

The cell lines HT-29 (human colorectal cancer cell line) and PC-3 (human prostate cancer cell line) used in this study were originally

obtained from the American Type Culture Collection (ATCC) and maintained according to the supplier's instructions.

The sulforhodamine B (SRB) assay[22] was used to examine the antiproliferative efficacy of ABZ and its derivatives. Cells plated in 96-well Corning tissue culture dishes at densities of 2,000–3,000 cell/well were left for 24 h at 37 °C under a humidified atmosphere containing 5% CO_2. Following attachment, cells were treated with cell culture medium (RPMI-1640 plus 5% fetal calf serum) containing various concentrations (0.01–5 μM) of ABZ or its derivatives. At the end of the treatment period (72 h), cells were fixed in 10% (w/v) trichloroacetic acid for 30 min at 4 °C followed by tap water washing (5×) and staining with 100 μl of 0.4% (w/v) SRB dissolved in 1% acetic acid. Unbound dye was removed by five washes with 1% acetic acid before air drying. Bound SRB was solubilized with 100 μl of 10 mM Tris base (pH 10.5) and the absorbance read at 570 nm. Each experiment was repeated twice. Results were normalized to vehicle treated cells (100% growth).

Synthesis

ABZ-amine (5-(propylthio)-1*H*-benzimidazol-2-amine). To 500 mg (1.88 mmol) of albendazole, 10 mL (0.18 mol) of 1,2-ethanediol and 20 mg of toluene-4-sulfonic acid were added, and stirred at 120 °C in an oil bath for 24 h. The mixture gave a yellow solution. The 1,2-ethanediol was removed *in vacuo*. 10 mL of dichloromethane was added and stirred at RT for several hours. The solid was filtered and dried in a drying pistol at 50 °C for one day to remove traces of ethanediol. The filtrate was evaporated to a quarter of the original volume. White crystals were collected by filtration and combined with the solid previously collected. Yield 235 mg (60%). Anal. Calc. for $C_{10}H_{13}N_3S$, C, 57.94%; H, 6.32%; N, 20.27%; Found: C, 57.58%; H, 6.65%; N, 20.08%, ^{1}H NMR (CD_3OD) δ (ppm) 0.99 (t, *J* 7.4 Hz, 3H, CH_3), 1.58 (Sextet, *J* 7.4 Hz, 2H, CH_2), 2.80 (t, *J* 7.4 Hz, 2H, CH_2), 7.05 (dd, *J* 8.1, 1.5 Hz, H, benz CH5), 7.09 (d, H, benz CH6), 7.24 (d, *J* 1.5 Hz, H, CH9); ^{13}C NMR (CD_3OD) δ (ppm) 13.5 ($CH_3CH_2CH_2S$), 22.5 ($CH_3CH_2CH_2S$), 36.9 ($CH_3CH_2CH_2S$), 112.3 (Ar), 113.8 (Ar), 124.5 (Ar), 127.9 (q Ar), 133.0 (q Ar), 134.8 (q, Ar), 153.3 (imidazole); and *m*/*z* 208 (M+1); υ_{max} 3390 (NH), 3143 and 3072 (NH), 2963, 1668, 1558, 1442, 1269 cm^{-1}.

MEABZ (2-methoxyethyl) 5-propylthio-1*H*-benzimidazole-2-yl carbamate. To 100 mg (0.48 mmol) of 5-propylthio-1*H*-benzimidazol-2-amine (ABZ-amine), 0.458 g (3.32 mmol) of K_2CO_3, 5 mL of DMF and 112 μL (0.90 mmol) of 2-methoxyethyl chloroformate were added and stirred under N_2 at RT for 2 days. The solid was removed by filtration. The filtrate was collected and DMF was removed *in vacuo*, leaving the crude product (75~80%). The final product was purified by silica gel chromatography. The product was eluted with a 2 : 1 diethyl ether : hexane solution. Yield 110 mg, 74%, m.p 149.1~150.5 °C; Anal. Calc. for C_{14} $H_{19}N_3O_3S$ C, 54.35%; H, 6.19%; N, 13.58%; S, 10.36% Found: C, 54.34%; H, 6.11%; N, 13.42%; S, 10.58%; ^{1}H NMR (CD_3OD) δ (ppm) 1.00 (t, *J* 7.4 Hz 3H, CH_3), 1.60 (Sextet, *J* 7.4 Hz 2H, CH_2), 2.86 (t, *J* 7.3 Hz, 2H, CH_2), 3.40 (s, 3H, OCH_3), 3.68 (m, 2H, CH_2 CH_2OCH_3), 4.37 (m, 2H, CH_2 CH_2OCH_3), 7.20 (dd, *J* 8.3, 0.6 Hz, H, Bz), 7.34 (dd, *J* 8.3, 1.7 Hz, 1H, Bz), 7.48 (dd, *J* 1.7, 0.6 Hz, 1H, Bz); ^{13}C NMR (CD_3OD) δ (ppm) 12.1 ($CH_3CH_2CH_2S$), 22.3 ($CH_3CH_2CH_2S$), 37.3 ($CH_3CH_2CH_2S$), 57.7 (OCH_3), 64.5 (ethyl), 70.2 (ethyl), 113.5 (broad, Bz), 116.3 (broad, Bz), 125.2 (Bz), 128.7 (q Bz), 148.01 (carbonate), 154.7 (imidazole); *m*/*z*, 309 (M$^+$, 23%), 233 (97), 204 (27), 191 (100), 160 (31), 45 (83); υ_{max} 3384 (NH), 2960-2445, 1707 (C=O), 1635, 1589, 1458, 1261 cm^{-1}.

HEABZ (2-hydroxyethyl) 5-propylthio-1*H*-benzimidazole-2-yl carbamate. Ethanediol was dried by azeotropic distillation. THF was added to the diol and continuously distilled through a column of activated molecular sieves overnight. This process was repeated twice with freshly activated molecular sieves. Finally the THF was removed by distillation.

0.45 g (1.7 mmol) of albendazole dried in an oven at 120 °C overnight, 5 mL of dried ethanediol and 0.02 g (0.12 mmol) of dried toluene-4-sulfonic acid were added into a 25 mL round flask, stirred at 135 °C in an oil bath for 9 h. The mixture developed a brown colour. The ethanediol was removed *in vacuo* at 95 °C, 3 mm Hg. The crude material contained 50% HEABZ. The residue was extracted with 30 mL of hot water five times. The hot solution was combined and cooled to RT. The white precipitate that developed was collected by filtration. The product was dissolved in a minimum volume of methanol and was loaded onto a column of alumina (2 cm × 15 cm). The product was eluted with methanol. Methanol was removed *in vacuo*. Yield 30 mg, 6%, m.p 107.1~108.1 °C; ^{1}H NMR (CD_3OD) δ (ppm) 0.99 (t, *J* 7.4 Hz, 3H, CH_3), 1.58 (sextet, *J* 7.4 Hz, 2H, CH_2) 2.83 (t, *J* 7.4 Hz, 2H, CH_2), 3.85 (t, *J* 5.1 Hz, 2H, CH_2), 4.10 (t, *J* 5.1 Hz, 2H, CH_2), 7.14 (d, *J* 1.6 Hz, H, Bz) 7.14 (d, *J* 0.8 Hz, 1H, Bz), 7.30 (s-broad, 1H, Bz); *m*/*z*, 296 (M$^+$+1, 98%), 290 (41), 274 (100); Found (HRMS): (M-C_2H_4O)$^+$, 251.1090. Calc. for C_{13} $H_{17}N_3O_3S$: (M-C_2H_4O)$^+$, 251.1092. υ_{max} 3340 (NH), 3228, 2958, 1650, 1635, 1541, 1458, 1474, 1076 and 1062 (C–O) cm^{-1}.

MEABZ regio-isomers A and B: N1-(2-methoxyethoxy-carbonyl)-2-amino-5-propylthiobenzimidazole; N1-(2-methoxy-ethoxycarbonyl)-2-amino-6-propylthiobenzimidazole. To 100 mg (0.48 mmol) of 5-propylthio-1*H*-benzimidazol-2-amine (ABZ-amine), 0.458 g of (3.32 mmol) K_2CO_3, 5 mL of acetone/methanol and 60 μL (0.48 mmol) of 2-methoxyethyl chloroformate were added, stirred under N_2 at RT for 3 h. The solid was removed by filtration. The filtrate was collected and the acetone/methanol was removed *in vacuo*, leaving the crude product. The crude product was dissolved in a minimum volume of acetone and loaded onto a silica gel column (2 cm × 10 cm). The products were eluted with a 1 : 3 diethyl ether : hexane solution. MEABZ isomer A eluted first, followed by MEABZ isomer B. MEABZ isomer A crystallized from the eluant, yield 10 mg, 7%, m.p. 117.6~120.8 °C; Anal. Calc. for $C_{14}H_{19}N_3O_3S$, C, 54.35%; H, 6.19%; N, 13.58%; S, 10.36% Found:. C, 54.00%; H, 6.07%; N, 13.27%; S, 10.12%; ^{1}H NMR (CD_3OD) δ (ppm) 0.99 (t, *J* 7.4 Hz 3H, CH_3), 1.60 (sextet, *J* 7.4 Hz, 2 H, CH_2), 2.86 (t, *J* 7.4 Hz, 2H, CH_2), 3.43 (s, 3H, OCH_3), 3.79 (m, 2H, CH_2 CH_2OCH_3), 4.61 (m, 2H, CH_2 CH_2OCH_3), 7.05 (dd, *J* 8.4, 1.8 Hz, 1H, Bz), 7.21 (d, *J* 1.8 Hz, 1H, Bz), 7.63 (d, *J* 8.4 Hz, 1H, Bz); *m*/*z* 332 (M + Na$^+$, 27%), 310 (M$^+$+1, 57), 266 (11), 208 (100), 59 (91); υ_{max} 3412 (NH), 1734 (C=O), 1321 cm^{-1}.

MEABZ isomer B crystallized from the eluant, yield 8 mg, 5%, m.p. 122.8~125.2 °C; Anal. Calc. for $C_{14}H_{19}N_3O_3S$, C, 54.35%; H, 6.19%; N, 13.58%; S, 10.36% Found: C, 54.35%; H, 5.91%; N, 13.27%; S, 10.21%.; ^{1}H NMR (CD_3OD) δ (ppm) 0.99 (t, *J* 7.4 Hz 3H, CH_3), 1.59 (sextet, *J* 7.4 Hz, 2 H, CH_2), 2.84 (t, *J* 7.4 Hz, 2H, CH_2), 3.47 (s, 3H, OCH_3), 3.81 (m, 2H, CH_2 CH_2OCH_3), 4.62

(m, 2H, *CH*$_2$ CH_2OCH_3), 7.13 (d, *J* 8.2 Hz, 1H, Bz), 7.25 (dd, *J* 8.2 Hz, 1.8 Hz, 1H, Bz), 7.85 (d, *J* 1.8 Hz, 1H, Bz); *m/z* 332 ($M + Na^+$, 16%), 310 (M^++1, 34), 266 (10), 208 (100), 59 (59); υ_{max} 3426 (NH), 3109-2808, 1735 (C=O), 1627, 1458, 1381, 1328, 1219 cm^{-1}.

Results

Synthesis of HEABZ and ABZ-amine

The amount of water in the ethanediol, the reaction temperature and the time were some of the variables responsible for the proportion of hydrolysis *versus* transesterification, leading to different ratios of HEABZ to ABZ-amine (Scheme 1).

A series of experiments were carried out to determine the effect of water in the reaction. Under wet conditions, the reaction mixture mainly contained ABZ-amine as determined by ^{1}H NMR in D_2O. The ratio of HEABZ was determined by comparing the integrals of the hydroxyl ethyl proton peaks at 3.62 ppm and 4.31 ppm against the propylthio methylene proton peaks at 0.94 ppm, 1.54 ppm and 2.80 ppm. The proportion of HEABZ can be optimized by careful drying of the ethanediol, the ABZ, and by using anhydrous toluene-4-sulfonic acid and a dry nitrogen atmosphere. The highest proportion of HEABZ that could be obtained was 50% (see Supplementary Information†). The amount of water in the reaction system determines the balance between the HEABZ and ABZ-amine. However, under anhydrous conditions, the ethanediol could also be responsible for the formation of ABZ-amine, through the formation of an alkylcarbonate. This was not tested, but observations made with changes in temperature also point to this conclusion.

Synthesis of MEABZ

The relative reactivity of the three nucleophilic nitrogen substitution positions of ABZ-amine was affected by the polarity of the solvent. Only MEABZ was observed when DMF was the solvent (Scheme 2).

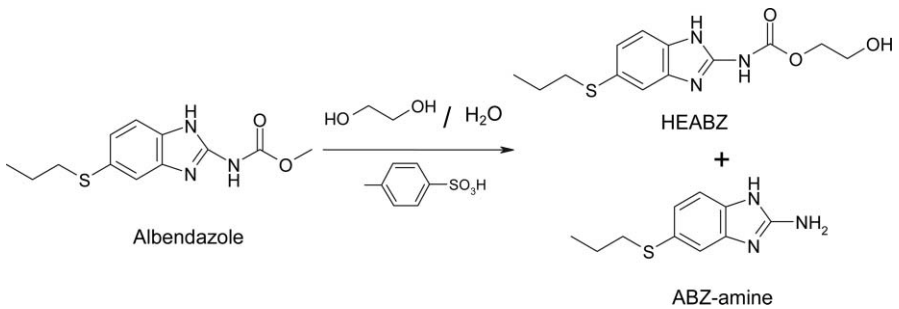
Table 1 Microanalysis results for MEABZ and the MEABZ isomers A and B

Required Elements	Calculated $C_{14}H_{19}N_3O_3S$	Found		
		MEABZ	(A)	(B)
C	54.35	54.34	54.00	54.35
H	6.19	6.11	6.07	5.91
N	13.58	13.42	13.27	13.27
S	10.36	10.58	10.12	10.21

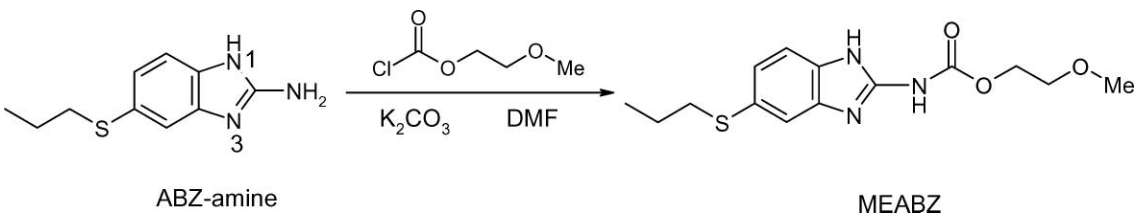
Compared to the parent molecule ABZ-amine, the product MEABZ has a methoxyethyl carbamate functional group. This main difference is seen in the ^{1}H and ^{13}C NMR spectra in CD_3OD, where the chemical shifts are δ 3.40 and 3.68 ppm for the ethylene protons and 4.37 ppm for methoxyl protons in the ^{1}H NMR and δ 57.7 ppm for methoxy, 64.5 and 70.2 ppm for ethylene and 148.01 ppm for the carbamate in the ^{13}C NMR. In support of the structure of MEABZ, the ^{13}C NMR spectrum shows a characteristic feature for the aromatic ring of ABZ – the peaks at 118 ppm and 120 ppm are broad due to the relatively slow exchange of the proton from the N1 to the N3 of the imidazole ring. These exchange broadened resonances are observed in the ^{13}C NMR spectrum of MEABZ with the peaks at 113.5 and 116 ppm, but not in the spectra of the MEABZ isomers A and B. Significantly, the M^+ ion, at *m/z* 309 in the mass spectrum and a peak at 1707 cm^{-1} for the carbamate stretch in the IR spectrum both support the MEABZ structure. In addition, the microanalysis agrees with the calculated empirical formula, shown in Table 1.

Synthesis of MEABZ isomers A and B

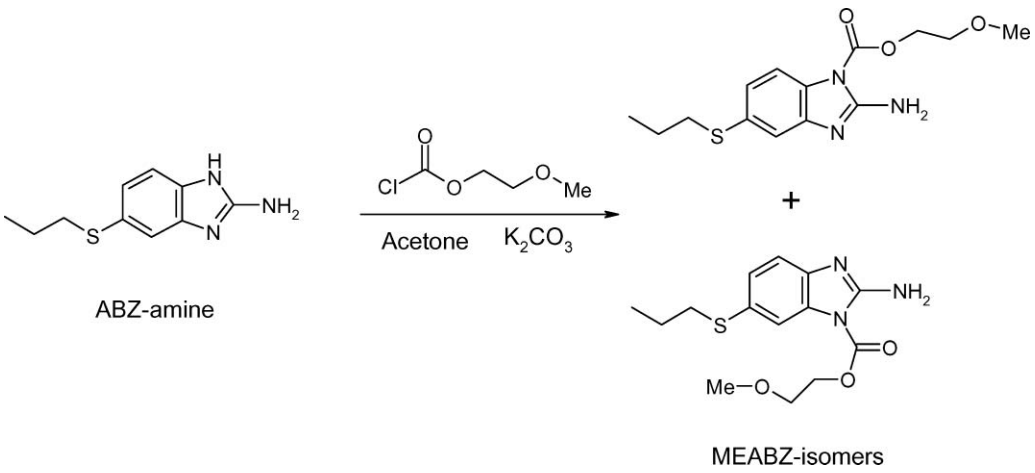
Two MEABZ regio-isomers, A and B, were observed when acetone was used as the solvent (Scheme 3). The ^{1}H NMR (in CD_3OD), microanalysis, IR and mass spectral data of isomers A and B indicated that they are isomeric with MEABZ. The major



Scheme 1 Synthesis of HEABZ and ABZ-amine.



Scheme 2 Synthesis of MEABZ.



Scheme 3 Synthesis of MEABZ isomers A (upper structure) and B (lower structure).

difference between the three isomers is seen in the ^{1}H NMR spectrum, where the chemical shifts of the aromatic protons for MEABZ are significantly different to isomer A and B. In addition, smaller but significant shifts are also observed for the methoxyethyl group protons of isomers A and B, compared to MEABZ. As noted before, the characteristic broad resonances for the aromatic ring carbons of MEABZ were not observed for the MEABZ isomers A or B in the ^{13}C NMR spectra. In addition, the existence of two pronounced M+Na$^+$ and M+1$^+$ ions, for ESMS *m/z* 332 and 310, for A and B supports the proposed structure for the regio-isomers. Similarly, the microanalysis results (Table 1) and the IR results of the three compounds were observed and these all strongly suggest isomeric structures.

Based on the comparison of the chemical shifts of the H4 and H7 resonances of MEABZ with the respective protons of isomers A and B, isomer A is assigned to N1-(2-methoxyethoxy-carbonyl)-2-amino-5-propylthiobenzimidazole, while isomer B is assigned to N1-(2-methoxyethoxycarbonyl)-2-amino-6-propylthiobenzimidazole. For isomer A, a large downfield shift of the H7 of MEABZ and a smaller upfield shift for H4 are observed, whereas, for isomer B the reverse pattern of chemical shift changes is seen.

Interestingly, the two regio-isomers A and B, appeared to be less stable than MEABZ in methanol at room temperature. For example, when the two isomers A and B and MEABZ were left in methanol solutions at room temperature for 3 days, significant changes occurred in the ^{1}H NMR spectra of the two isomers, with new resonances from the aromatic and methoxyethyl protons observed. In the ^{1}H NMR spectrum of isomer B, the integral of the proton at δ 7.85 ppm decreased to 37% in three days (see Supplementary Information†). New resonances in the aromatic region consistent with ABZ-amine were observed. In addition, new peaks at δ 3.25, 3.62 and 4.25 ppm appeared, consistent with the solvolysis product methoxyethanol. All the observed changes strongly suggest that isomer B degraded to ABZ-amine in methanol. A series of ^{1}H NMR experiments showed that isomer A has a similar solvolysis rate to isomer B under the same conditions. In contrast, MEABZ shows greater stability under the same conditions, with <1% degradation product observed after three days in methanol.

In vitro cytotoxicity

The cytotoxicity of the ABZ derivatives was tested against human colorectal cells (HT-29) and human prostate cancer cells (PC-3). The results (Fig. 2 and Fig. 3) show that MEABZ is effective in inhibiting the proliferation of this set of cells. Furthermore, in these cell lines, MEABZ is 5~10 times more active than the parent ABZ. The IC_{50} values of MEABZ are 0.02 and 0.08 μM in the HT-29 and PC-3 cell lines respectively. In contrast, ABZ-amine, HEABZ and the MEABZ regio-isomers A and B had no effect on the proliferation of these cells (Fig. 2 and Fig. 3).

The cytotoxicity of MEABZ encapsulated in either Q[7] or Q[8] was also examined. The results (see Fig. 4) show that encapsulation does reduce the *in vitro* cytotoxicity of MEABZ, but only to a small degree. The encapsulated MEABZ is still significantly more active than the parent drug ABZ. These results are consistent with our previous study where the cytotoxicity of ABZ was slightly reduced by encapsulation in Q[7].

Cucurbit[*n*]uril binding for MEABZ

Fig. 5 shows the ^{1}H NMR spectra of MEABZ in D_2O with added Q[6], Q[7] and Q[8] at pH 3.5, the pH obtained upon mixing MEABZ with Q[*n*]. Importantly, the addition of the Q[*n*] solubilised the MEABZ. In control experiments, the aqueous solubility of MEABZ at pH 6.6 was found to be 8 (± 0.5) μM and 18 (± 1) μM at pH 3.5. The maximum solubility obtainable with Q[6,7,8] is summarised in Table 2. When the pH of a MEABZ@Q[7] sample prepared at pH 3.5 was raised to 6.6, the aqueous solubility of MEABZ decreased from 7.3 mM to 5.0 mM. No dramatic decrease of the solubility was observed for a MEABZ@Q[8] sample when the pH of the sample was raised to 6.6. The maximum solubility of MEABZ (9.4 mM) was obtained with Q[8] (Table 2).

As can be seen in Fig. 5, a considerable difference can be observed in the chemical shift of the resonances from MEABZ when solubilised by Q[6] compared to that with Q[7] and Q[8] (Table 3). Fig. 5 shows MEABZ with added Q[6] at a 3 : 1 molar ratio. Two sets of resonances from MEABZ are observed for the methoxyethyl protons, indicating slow exchange (on the NMR

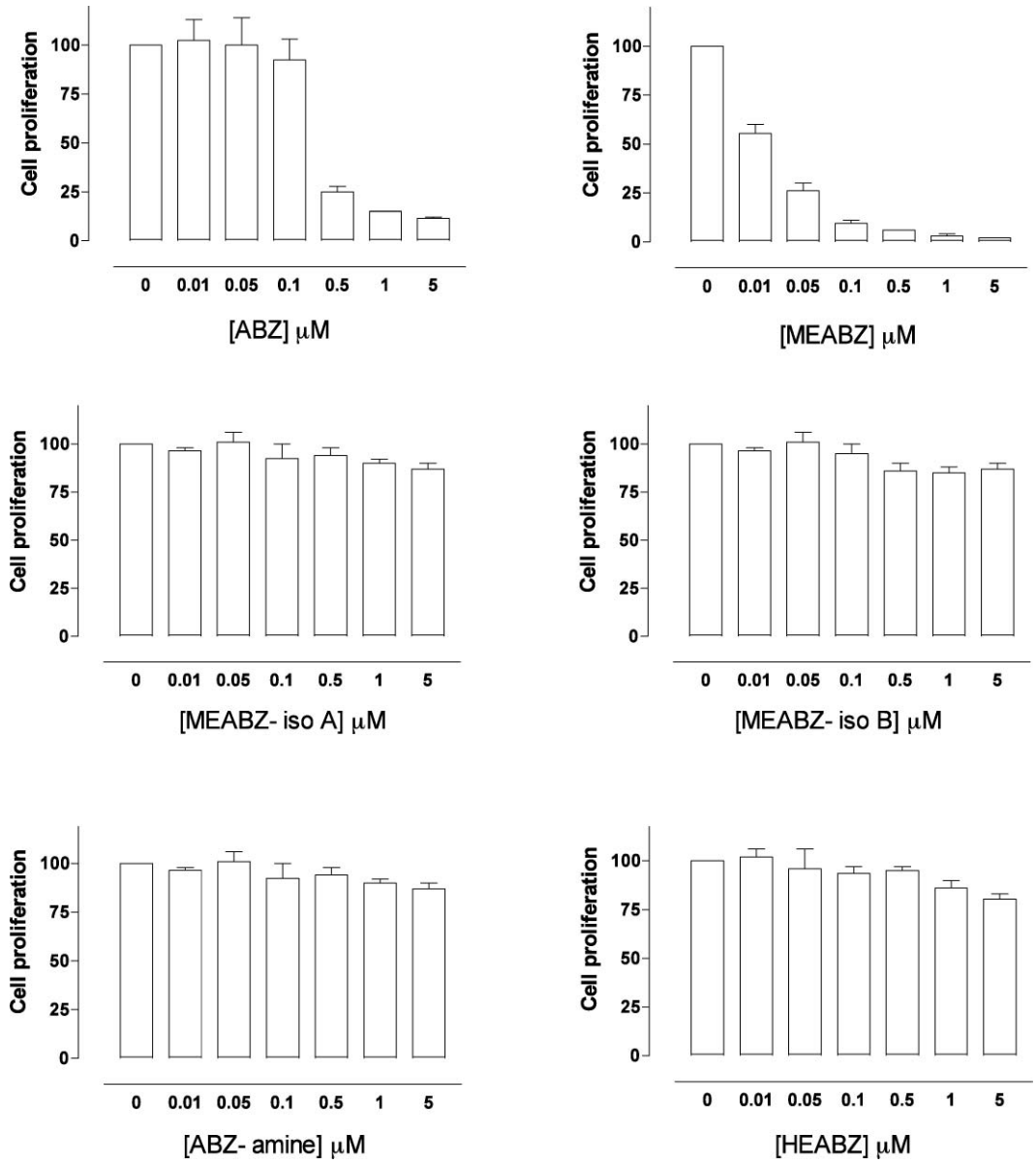


Fig. 2 Proliferation of HT-29 cells in culture medium containing various concentrations (0.01–5 μM) of ABZ or its derivatives. Following 72 h of treatment, cell proliferation was measured by SRB assay and results (mean ± s.e.m.) are expressed as % of control.

time scale) between 'free' (portal bound) and Q[6]-encapsulated MEABZ forms. One set of methoxyethyl resonances show large upfield shifts, from what is expected. In addition, with the exception of the methoxyethyl group, all the MEABZ resonances are significantly downfield in the spectrum with added Q[6], compared to the corresponding peaks in the spectra with Q[7] and Q[8]. All the aromatic and propyl resonances became broad upon the addition of Q[6]. It has been established that resonances from guest protons that are located inside the cucurbituril cavity shift upfield.[20,23,24] The protons that are positioned near the centre of the host cucurbituril cavity are expected to exhibit the largest upfield shifts.[20,23,24] In comparison, small down field shifts are observed for resonances from guest protons located close to but outside of the cucurbit[*n*]uril portal. Based on the ^{1}H NMR, it was concluded that the methoxyethyl group of one MEABZ is positioned within the Q[6] cavity. The other set of the methoxyethyl resonances are broad, and no large changes in chemical shift were observed, compared to those observed with added Q[7,8]. Moreover, the peaks for the methylene protons of Q[6] that project towards the portals (5.75 ppm) are broadened, which suggests MEABZ strongly associates with the portal. Based on the chemical shifts and comparison of the integrals of the resonances of MEABZ (both portal bound and encapsulated parts) with those of Q[6], it is concluded that three MEABZ molecules interact with one Q[6] host molecule. One of the MEABZ, at least in part, is positioned inside the Q[6] cavity, while the other two MEABZ molecules are interacting with the Q[6] portals in an undetermined manner.

Fig. 5 shows MEABZ in D_2O with added Q[7] and Q[8] at a 0.7 : 1 and a 0.9 : 1 molar ratio, respectively, at pH 3.5. From the observed upfield shifts of MEABZ proton resonances with added Q[7] and Q[8], relative to that observed with Q[6] binding and free MEABZ in CD_3OD (see Table 3), it is concluded that the MEABZ

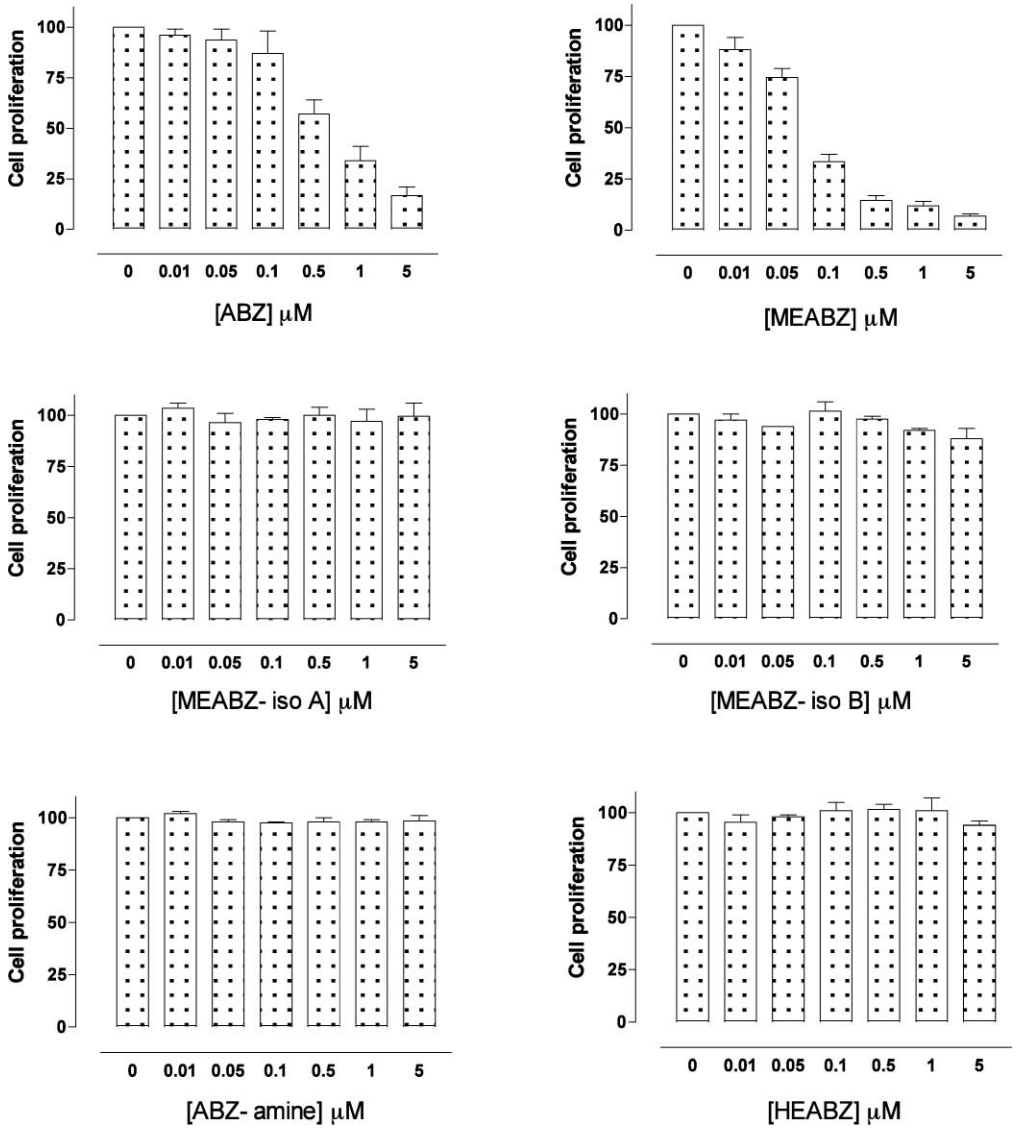


Fig. 3 Proliferation of PC-3 cells in culture medium containing various concentrations (0.01–5 μM) of ABZ or its derivatives. Following 72 h of treatment, cell proliferation was measured by SRB assay and results (mean ± s.e.m.) are expressed as % of control.

Table 2 Maximum solubility (mM) of MEABZ encapsulated in cucurbit[*n*]uril in D_2O at pH 3.5 at 25 °C

Cucurbit[*n*]uril	MEABZ:Q Mixed Ratio [a](MR)	MEABZ:Q Solution Ratio[b]	MEABZ Solubility/mM[c]
6	1	2.9	2.4
7	0.5	0.37	4.7
7	1	0.73	5.7
7	2	0.76	7.3
8	0.5	0.86	3.9
8	1	0.89	4.1
8	2	0.94	9.4

[a] Solid MEABZ (3.1 mg) and the appropriate proportion of Q[*n*] were combined with D_2O (1.0 mL) at 20 °C, shaken, sonicated and set aside for 20 h. [b] Resultant mixtures were filtered and the ratios determined by ^{1}H NMR at 25 °C using standardised EtOH/D_2O solutions, as indicated in the experimental. [c] Concentrations of MEABZ were determined by the same method as in b.

propyl and aromatic protons are located within the Q[7] or Q[8] cavity. In support, the methoxyethyl resonances have chemical shifts similar to what would be expected for this functional group if the methoxyethyl is positioned outside of the cucurbituril cavity. As the aromatic resonances from MEABZ exhibit differences in chemical shift upon Q[8]-binding compared to Q[7]-binding (Table 3), it is also concluded that the drug is positioned within the cavities of Q[7] and Q[8] in slightly different orientations. The large upfield shifts of the propyl methyl and aromatic resonances in the spectra of MEABZ with Q[7] and Q[8] indicates that both these groups must be positioned deep within the cucurbituril cavity. This could occur for MEABZ if the propyl chain folds back into the cavity that also contains the aromatic ring system. The larger cavity volume of Q[8], compared to Q[7], would then allow for different binding orientations.

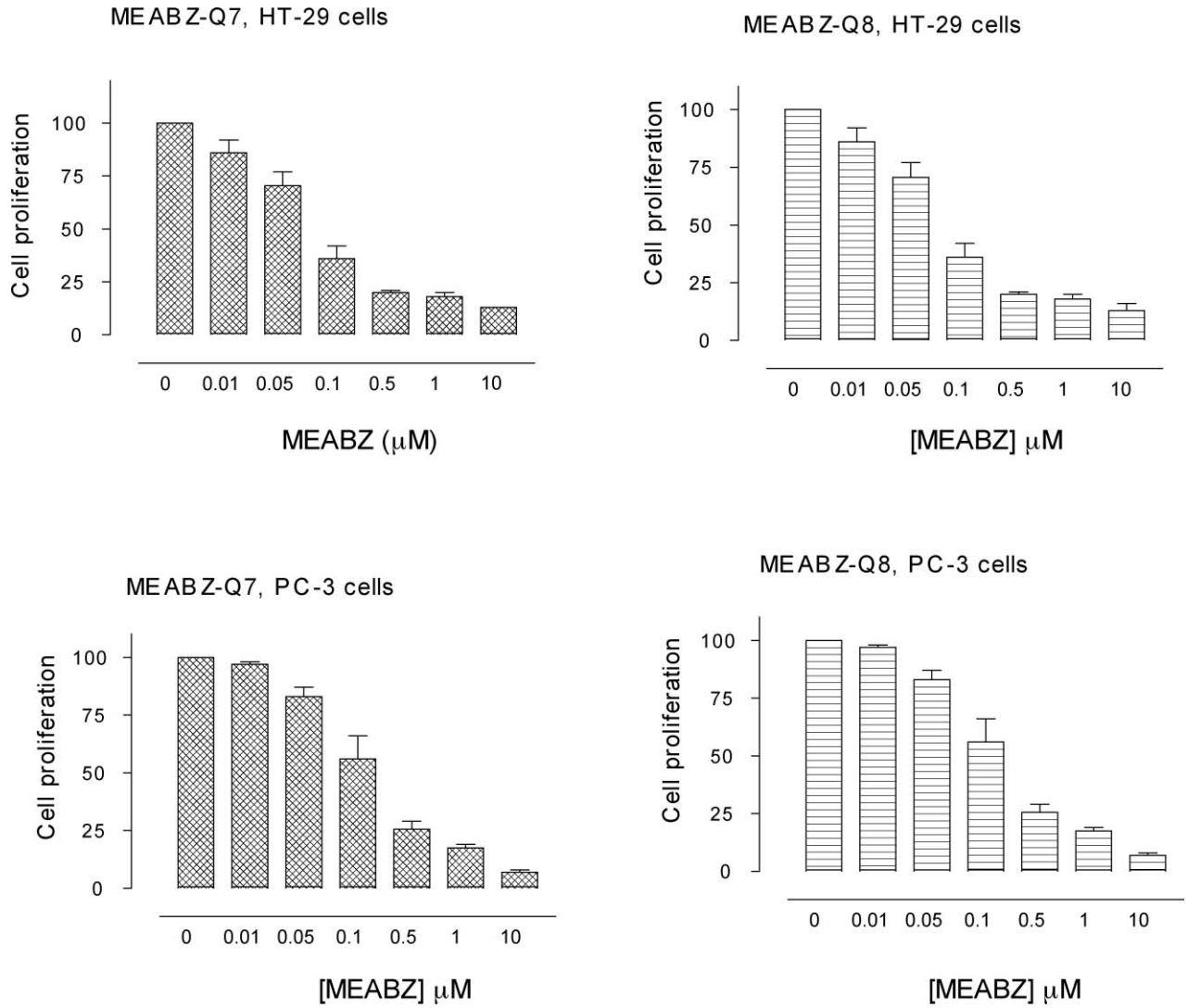


Fig. 4 Proliferation of HT-29 cells and PC-3 cells in culture medium containing various concentrations of MEABZ encapsulated in either Q[7] or Q[8]. Following 72 h of treatment, cell proliferation was measured by SRB assay and results (mean ± s.e.m.) are expressed as % of control.

Table 3 Chemical shifts of the non-exchangeable proton resonances of MEABZ and MEABZ encapsulated in Q[6,7,8]

Chemical Shift (ppm)					
Proton	MEABZ in CD_3OD[b]	MEABZ@Q[6] in D_2O		MEABZ@ Q[7] in D_2O	MEABZ@ Q[8] in D_2O
S-propyl					
CH_3	1.00	0.84		–0.03	0.10
CH_2	1.60	1.50		0.87	0.83
CH_2	2.86	3.01		2.25	2.32
Aromatic					
H4	7.48	[a]		6.70	6.94
H6	7.34	[a]		6.67	6.78
H7	7.20	[a]		7.40	6.67
Carbamate		**Portal**	**Encapsulated**		
CH_2	3.68	3.81	2.80	3.82	3.73
CH_2	4.37	4.52	3.54	4.53	4.45
CH_3	3.40	3.43	2.94	3.44	3.39

[a] Two sets of proton resonances are overlapped, and could not be individually assigned. [b] An NMR spectrum of free MEABZ in D_2O could not be obtained due to its extremely low solubility.

Determination of the pK_a of MEABZ

The pK_a value for the acid dissociation of free MEABZ was found to be 3.5. By contrast, pH titrations of the MEABZ encapsulated in either Q[7] or Q[8] yielded a pK_a value of 5.5 (see Supplementary Information†), giving a ΔpK_a of 2.0.

Discussion

In vitro cytotoxicity assays demonstrated that MEABZ, a new benzimidazole, was active against human colorectal (HT-29) and human prostate cancer (PC-3) cells at sub-micromolar concentrations, and interestingly, MEABZ is 5–10 times more active than the parent ABZ. In addition, MEABZ was found to be slightly more soluble in water at pH 6.6 than ABZ (8.5 μM *versus* 3.0 μM, respectively). Alternatively, ABZ-amine, HEABZ and MEABZ isomers A and B, were inactive. The lack of activity for the MEABZ isomers A and B may be due to their rapid degradation to the inactive ABZ-amine. This proposal is supported by the

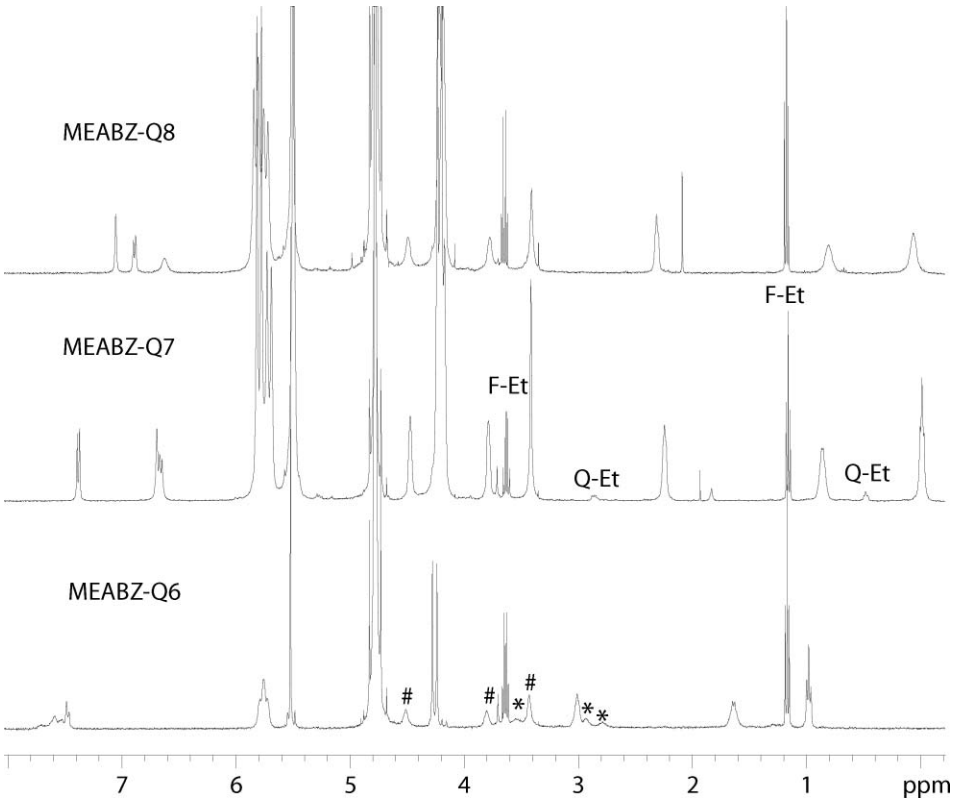


Fig. 5 ^{1}H NMR spectra of MEABZ in D_2O with Q[6], Q[7] and Q[8] at MEABZ to Q[*n*] ratios of 2.9, 0.7 and 0.9, respectively, containing 3.05 mM of added ethanol at 25 °C at pH 3.5. F-Et denotes free ethanol, while Q-Et indicates ethanol bound in the cucurbit[7]uril. # denotes free MEABZ, while * indicates MEABZ bound in the cucurbit[6]uril.

NMR experiments that showed the MEABZ isomers significantly degraded in 3 days by solvolysis in methanol.

Q[*n*] binding

Compared to ABZ binding,[10] MEABZ displays similar binding characteristics with Q[7] and Q[8]. The cucurbituril cavity is not deep enough to encapsulate both the propyl and aromatic groups, if the propyl-arm projects away from the aromatic system. The methyl group from the propyl-arm shifts further upfield than either of the methylene protons in the Q[7]- and Q[8]-bound forms, compared to their respective chemical shifts in the spectrum of the drug with added Q[6] where the propyl group is located outside of the cavity. This strongly suggests that the propyl-arm folds back into the cavity (as was shown for ABZ with Q[7,8]),[10] with the methyl group being positioned towards the centre of the cucurbituril cavity. The larger cavity of Q[8] allows more room for the propyl-arm to fold, and thereby allows the MEABZ to bind in a slightly different orientation compared with Q[7].

The aqueous solubility of MEABZ was significantly increased by encapsulation in Q[*n*]. Noticeably, the encapsulation in Q[8] increased the solubility of MEABZ by 3,000-fold, when compared to the solubility of free ABZ in water. When compared to MEABZ itself, encapsulation of MEABZ in Q[6] increases the water solubility by 200 to 300-fold at pH 3.5 and pH 6.6, respectively. Encapsulation in Q[7] and Q[8] increases the water solubility of MEABZ by 400- to 500-fold at pH 3.5 and 600- to 1200-fold at pH 6.6. The smaller solubility enhancement at pH 3 is due to the higher solubility of the free drug at this pH. MEABZ would be partially protonated at pH 3, based upon the determined pK_a of 3.5, and hence, would be more soluble in an aqueous solution than at pH 6.6.

Interestingly, the pK_a of MEABZ encapsulated in Q[7,8] increased by 2 pK_a units compared to the free drug. While this is a significant increase, larger changes in pK_a have been reported for other compounds upon Q[*n*] encapsulation.[25] Given the increase in pK_a upon Q[*n*]-binding, encapsulated MEABZ would be fully protonated at pH 3.5 and consequently the binding would be stabilised by stronger ion–dipole interactions at the Q[*n*] portal, compared to that at pH 6.6 where MEABZ would not be positively charged. This may account for the observation that for MEABZ@Q[7] samples prepared at pH 3.5 but then raised to pH 6.6, the aqueous solubility decreased from 7.3 mM to 5.0 mM. No solubility decrease was observed for MEABZ@Q[8] samples when the pH was raised from 3.5 to 6.6. This may indicate that the MEABZ@Q[8] is more stable than MEABZ@Q[7], or that the competitive binding of the added Na^+ is significantly stronger for Q[7] compared to Q[8]. However, and more importantly, the ability to maintain the higher solubility is physiologically relevant to the potential application of MEABZ as an anticancer agent.

Conclusion

In conclusion, a new benzimidazole carbamate drug (MEABZ) has been synthesised and shown to be significantly more cytotoxic than the parent compound ABZ, which is currently in trial as

an anticancer agent. Furthermore, the fact that another similar ABZ derivative, HEABZ, showed no cytotoxicity, highlights the sensitivity of the carbamate moiety of ABZ for anticancer activity. Although MEABZ was not significantly more water soluble than ABZ, encapsulation in Q[7,8] did significantly increase its solubility, as was previously shown for ABZ.[10] As encapsulation in Q[7] and Q[8] did not significantly reduce the cytotoxicity of MEABZ, Q[*n*] encapsulation may provide a formulation method for any clinical application that may arise for MEABZ.

Acknowledgements

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