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New biophysical probes for structure–activity analyses of vacuolar-H^+-ATPase enzymes †

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New EPR spin labelled and photoactivatable molecules have been designed to probe specifically the vacuolar-H^+-ATPase.

The vacuolar H^+-ATPases (V-ATPase) are ATP-driven proton pumps found in virtually all cells.[1] Although the primary function of these membrane proteins is to acidify intracellular compartments, in certain cell types they also act to extrude acid across the cell membrane.[2] This latter specialisation is the key to their involvement in processes such as bone metabolism and tumour cell invasiveness. The V-ATPase is therefore implicated in disease states such as osteoporosis and metastatic cancer,[3] and consequently continues to provoke interest as a potential drug target.

The naturally occurring plecomacrolides bafilomycin and concanamycin (Fig. 1) have been shown to be both potent and selective inhibitors of the V-ATPase, with IC_{50} values of 1 and 2 nM, respectively, against chicken osteoclast (cOc) cells.[4] Unfortunately, the twin issues of synthetic accessibility and extremely low toxicity thresholds make these plecomacrolides less than favourable as drug candidates. Nevertheless, the search for new and synthetically more accessible inhibitors has benefited greatly from extensive structure–activity studies on both bafilomycin and concanamycin.[4,5] The most promising of the synthetic products has been 5-(5,6-dichloro-2-indolyl)-2-methoxy-*N*-(1,2,2,6,6-pentamethylpiperidin-4-yl)-2,4-pentadienamide (Fig. 1c) developed by Farina *et al.*, which has been found to show inhibitory capability against chicken osteoclast V-ATPase in the high nM range.[6] Despite these advances it is still unclear how or where both the natural products and synthetic mimics carry out their inhibitory action. This has led recently to the development of structural probes for biophysical analysis of inhibitor binding, including photoactivatable derivatives of both the plecomacrolides[7] and subsequently the indoles.[8] The general consensus at present is that both macrolide antibiotics and the indole-based inhibitors bind to the proteolipid V_o complex, embedded within the vacuolar membrane (Fig. 2). Given the current lack of availability of high resolution structural data, information on binding site profile(s) is at a premium and biophysical tools can offer considerable and valuable data.

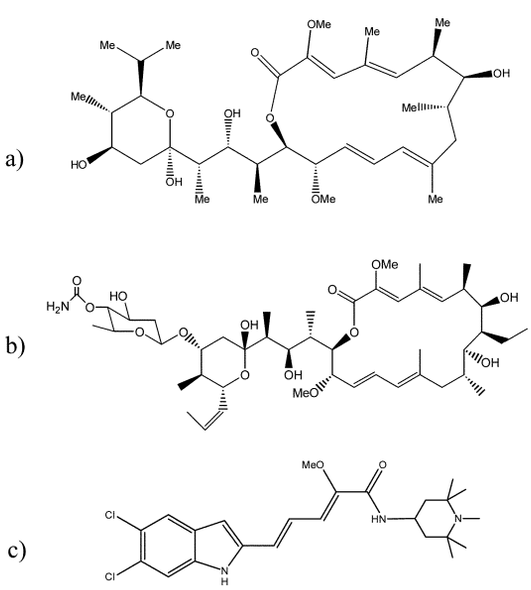

Fig. 1 (a) Bafilomycin A_1; (b) Concanamycin A; (c) 5-(5,6-Dichloro-2-indolyl)-2-methoxy-*N*-(1,2,2,6,6-pentamethylpiperidin-4-yl)-2,4-pentadienamide.

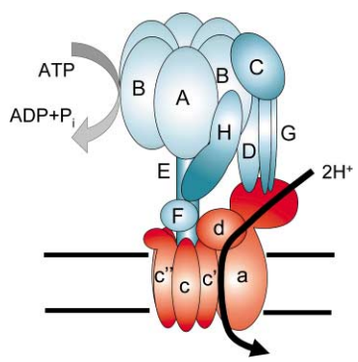

Fig. 2 Schematic structure of the V-ATPase enzyme complex.

Here we report our preliminary studies on new structural and synthetic probes of the 5-(2-indolyl)-2,4-pentadienoyl family which exploit two key elements; electron spin labels and photoactivatable labels.

The compounds **1** and **2** have been synthesised from 5-(5,6-dichloro-2-indolyl)-2-methoxy-2,4-pentadienoic acid[9] using amide coupling techniques (Scheme 1)‡ and both have been demonstrated to inhibit the yeast V-ATPase.

We were especially interested by the potential of spin-labelled derivative **1** because the TEMPO radical moiety is a close structural cousin to the indole of Fig. 1c. We envisaged that electron paramagnetic resonance (EPR) spectroscopic studies of **1** in both synthetic and vacuolar membranes would reveal important information concerning the location and orientation of this

† Electronic supplementary information (ESI) available: synthesis and characterisation details. See http://www.rsc.org/suppdata/ob/b3/b311401e/

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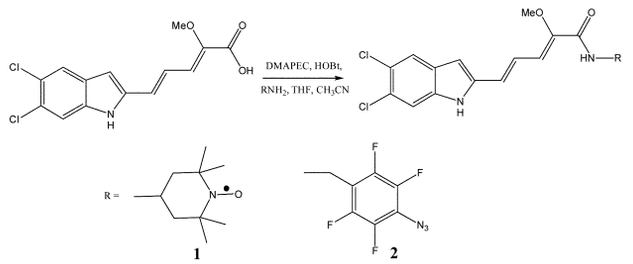

Scheme 1 Synthesis of compounds **1** and **2**.

class of indole inhibitor within the cell membrane. Furthermore, from a deconvolution analysis of the EPR line-shape, we envisaged that we should be able to establish whether or not the indole is interacting directly with transmembrane, receptor proteins.

Compound **1** displays the environmentally sensitive EPR properties required of an effective spin-label probe. Fig. 3 shows the EPR spectra of **1** in bilayer membranes composed of the lipid dimyristoyl phosphatidylcholine (DMPC), and also freely tumbling in chloroform : methanol solution. The spectral line shapes clearly indicate progressive motional averaging of the anisotropy in the ^{14}N-hyperfine splitting, from the top spectrum to the bottom spectrum in Fig. 3. The bottom spectrum, for the nitroxide free in solution, is the characteristic three-line, isotropic hyperfine pattern for $I = 1$.

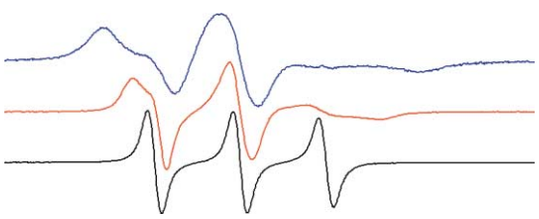

Fig. 3 9 GHz EPR spectra of compound **1** (1 mol%) in dimyristoyl phosphatidylcholine membranes; (top) 6 °C; (middle) 40 °C; (bottom) in $CHCl_3$: MeOH (2 : 1 v/v) solution (*ca.* 0.4 mM) at room temperature. Total display width = 100 G.

The temperature dependence of the motional averaging from **1** in membranes is quantified in Fig. 4 by using the maximum line height of the EPR spectrum, normalised to the total (double-integrated) spectral intensity. The response to the cooperative chain-melting phase transition of DMPC membranes, at 23–24 °C, demonstrates not only that the compound **1** is integrated into the hydrophobic interior of the membrane, but also that this molecular probe is a good tool for the observation of any possible ligand–protein interactions in membranes.

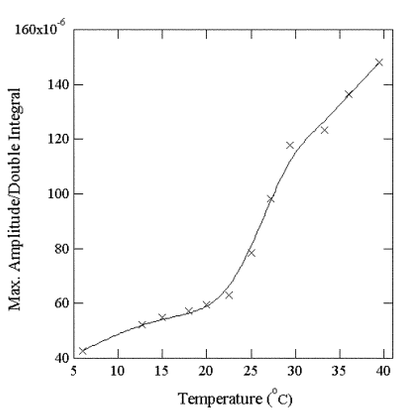

Fig. 4 Temperature dependence of the maximum EPR-amplitude of compound **1** in DMPC membranes. The EPR-amplitude is normalised to total spin intensity by using the double integral of the conventional first-derivative spectrum.

Furthermore, analysis of relaxation behaviour of **1** within DMPC in the presence of the hydrophobic and hydrophilic relaxation enhancers, oxygen and $NiSO_4$ respectively, confirms that the molecular probe is partitioned effectively within the membrane. Subsequently, compound **1** was also found to incorporate successfully within yeast vacuolar membranes, and preliminary analysis indicates that there are distinct changes in line-shape of the EPR signal, compared with artificial membranes, which is indicative of interaction with membrane proteins. A detailed report of its EPR behaviour in this and related membranes will appear in a full paper.

Compound **2** is potentially a valuable structural probe for photo-affinity labelling (PAL) investigations of the inhibitor binding site of the V-ATPase. In preliminary studies, 50 μM concentrations of **2** were incubated with detergent-solubilised V-ATPase isolates.[10] Subsequent to irradiation at 300 nm for 2 minutes, the affinity purified membrane domain of the enzyme[11] showed fluorescence labelling diagnostic for covalent attachment of the fluorophore of **2** (Fig. 5). This labelling was dependent on UV irradiation, and was partially blocked by pre-incubation with 50 μM concanamycin A.§

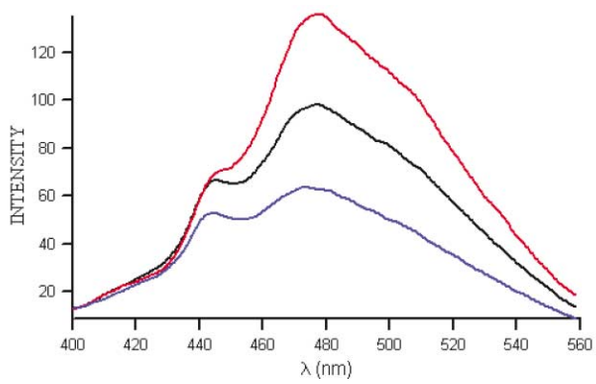

Fig. 5 Fluorescence emission spectra of compound **2**; (top) 50 μM of **2** + UV irradiation; (middle) 50 μM of **2** dark; (bottom) 50 μM of Concanamycin + 50 μM of **2** + UV irradiation.

In conclusion, we have demonstrated the potential of two new, complementary molecular probes for the analysis of inhibitor binding to the vacuolar ATPase enzyme. EPR spectroscopic studies on compound **1** clearly indicate incorporation within membranes, whilst preliminary photoaffinity experiments with compound **2** suggest that members of the 5-(2-indolyl)-2,4-pentadienoyl family of V-ATPase inhibitors target the transmembrane, proteolipid component of the enzyme.

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Notes and references

‡ **1**: (2*Z*,4*E*)-5-(5,6-Dichloro-2-indolyl)-2-methoxy-2,4-pentadienoic acid[3b] (100 mg, 0.32 mmol), 1-hydroxybenzotriazole, (48 mg, 1.1 eq.), and *N*-[3-(dimethylamino)-*N*-ethylcarbodiimide hydrochloride (68 mg, 1.1 eq.) dissolved in CH_3CN (3 ml) and THF (1 ml) were heated at 40 °C under N_2 for 1 h. After which the mixture was warmed to 60 °C, 4-amino-2,2,6,6-tetramethylpiperidinoxy free radical (66 mg, 1.2 eq.) added and then refluxed for 1 h. After cooling the solvent was removed under reduced pressure, the residue treated with 10% NaOH, extracted (EtOAc), dried ($MgSO_4$) and concentrated to give crude product. Purification by flash column chromatography eluted with EtOAc : Petrol (7 : 3) to give (2*Z*,4*E*)-5-(5,6-dichloro-2-indolyl)-2-methoxy-*N*-[4-(2,2,6,6-tetramethylpiperidinoxy)]-2,4-pentadienamide (118 mg, 79%) as a yellow crystalline solid, R_f 0.47 (EtOAc : Petrol, 7 : 3); ν_{max}/cm^{-1} 3054 (NH), 2987 (CH), 1610 (C=O); δ_H (300 MHz, DMSO) 11.85 (1 H, bs, NH), 7.81 (1 H, bs, ArH), 7.59 (1 H, bs, ArH), 7.26–6.95 (3 H, bm, 3CH), 6.66 (1 H, bs, ArCH), 4.08 (1, bs, CH), 3.80 (3, bs, OCH_3), 3.72–3.44 (16 H, bm); *m*/*z* (ES) 464.1501 (100%, M^+ $C_{23}H_{28}N_3O_3Cl_2$ requires 464.1508).

2: A solution of (2*Z*,4*E*)-5-(5,6-dichloro-2-indolyl)-2-methoxy-2,4-pentadienoic acid[3b] (70 mg, 0.22 mmol), 1-HOBt (33 mg, 1.1 eq.), DMAPEC (46 mg, 1.1 eq.) in acetonitrile (2 ml) and THF (0.8 ml) was heated at 40 °C for 90 min, the temperature was raised to 60 °C and *p*-azido-tetrafluorobenzyl amine[12] (73 mg, 1.5 eq.) dissolved in THF (1 ml) was added. The mixture was heated at reflux for 1 h, cooled, treated with 2 M NaOH and extracted with EtOAc, dried ($MgSO_4$) and evaporated to dryness. The crude was purified by flash column chromatography with gradient elution THF : hexane, 1 : 1, to THF : MeOH, 9 : 1, to give the pure target molecule (52 mg, 48%) as a brown oil, R_f 0.30 (THF : hexane); ν_{max}/cm^{-1} 3331 (NH), 2926 (CH), 2855 (CH), 2121 (N_3) 1656 (*CO*NH); δ_H (300 MHz, $CDCl_3$) 8.97 (1 H, bs, NH), 7.52 (ArH), 7.29 (1 H, s, ArH), 6.96–6.92 (1 H, m, CH), 6.85–6.78 (2H, m, CH + ArH), 6.65–6.64 (1 H, m, CH), 6.45 (1 H, bs, NH), 4.55 (2 H, d, *J* 5.9. CH_2), 3.69 (3 H, s, OCH_3); δ_F (188.3 MHz, $CDCl_3$) 143.4, 151.6; *m*/*z* (ES) 488.0526 (100%, $M^{+\bullet} - N_2 + H_2$, $C_{21}H_{15}Cl_2F_4N_3O_2$ requires 488.0556).

§ Detergent-solubilised V-ATPase isolated by glycerol gradient centrifugation.[10] After labelling, the membrane domain was extracted by affinity chromatography using the His6 tag on the proteolipid component interacting with Ni–NTA agarose support. Labelled protein eluted with 300 mM imidazole,[11] and the fluorescence yield was normalised to protein concentration.

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