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Synthesis of Sulfonamide Conjugates of Cu(II), Ga(III), In(III), Re(V) and Zn(II) Complexes: Carbonic Anhydrase Inhibition Studies

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Carbonic anhydrase IX (CA IX) is currently generating great interest as a marker of tumour hypoxia and a potential chemotherapeutic target. In order to test the principle that a CA IX inhibitor could be used for targeting PET or SPECT metallic radioisotopes to tumours we have prepared a number of conjugates involving aryl-sulfonamides or an acetazolamide derivative linked to a range of copper, indium, rhenium, 99m-technetium and zinc complexes. Radiolabelled ^{64}Cu and ^{99m}Tc analogues of the 'cold' Cu and some of the Re complexes were prepared in good radiochemical incorporation. Inhibition of various human carbonic anhydrase isoforms (I, II, IX and XII) was tested with the 'cold', non-radiolabelled complexes, and compared with an acetazolamide standard (AZA). The molecular structure of a new, tri-sulfonated porphyrin-labeled sulfonamide was determined using synchrotron X-ray crystallography.

Introduction

Carbonic anhydrases (CA) are zinc metalloenzymes that catalyse the interconversion between CO_2 and HCO_3^-, maintaining pH balance in blood plasma and transporting carbon dioxide out of tissue. Sixteen isoforms of carbonic anhydrase exist to date, located in the cytosol, mitochondria and cellular membranes.[1] Four transmembrane isozymes are implicated in the control of cell proliferation and cellular transformation. Particular interest surrounds the transmembrane CA IX isozyme, which has been shown to be overexpressed in certain cancer lines including renal, cervical squamous, ovarian, colorectal, bladder and non-small cell lung carcinoma. Strong association between CA IX expression and intra-tumoural hypoxia has also been demonstrated.[2] At a molecular level the response to hypoxia is the stabilization and activation of the transcription factor HIF-1α and its targets. Under normoxic conditions HIF-1α is hydroxylated and binds the ubiquitin ligase von Hippel –Lindau (VHL) protein, targeting the HIF-1α for degradation. Loss or mutation in VHL triggers the hypoxic phenotype. The tumour associated upregulation of CA IX is the result of a strong transcriptional activation of the CA9 gene by HIF-1α.[3] Studies on non-small cell lung carcinomas have shown CA IX association with proteins involved in angiogenesis, apoptosis inhibition and cell-cell adhesion disruption suggesting a strong relationship of this enzyme to a poor clinical outcome.[4] Therefore, current research interest focuses in the development of CA inhibitors as an approach to the treatment of cancers where CA IX is over- expressed. Similar interest surrounds CA XII, a second tumour associated CA isozyme which has been identified in renal cell carcinoma.[5] It

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is similarly regulated by tumour hypoxia but by different biochemical pathways to the CA IX isozyme.[3] Although it is less effective as a catalyst for CO_2 hydration compared to CA IX, its catalytic activity is associated with the acidification of the tumour environment under hypoxia. The inhibition of these tumour associated CA isozymes could represent a novel therapeutic approach for the management of hypoxic tumours which over- express these proteins.[6,7]

Selectivity for CA IX over the other CA enzymes has been investigated through docking studies / molecular modeling and X-ray crystal structure analysis[8] and it was found that the guanidine moiety of an arginine residue is specific to CA IX enzyme, which also has a larger active site than the CA I and CA II isozymes.[8] Inhibition of CA by arylsulfonamides was a matter of intense investigation and the Supuran group has carried out some comprehensive screening studies on libraries of sulfonamides to find the most selective compounds for CA binding. Inhibition studies with a series of aromatic and heterocyclic sulfonamides showed that CA IX and CA XII are sulfonamide avid.[9,10] 4-(2-Aminoethyl) benzenesulfonamide (**ABS**) was shown to be an efficient and specific CA IX inhibitor with an approximate selectivity of more than 60 fold for CA IX over the CA I isozyme. Another sulfonamide derivative acetazolamide (**AZA**), a known inhibitor of CA IX, is a clinically approved drug (Diamox)[11] with a strong binding affinity for CA IX and CA XII in comparison with the CA I and CA II enzymes.[3,9]

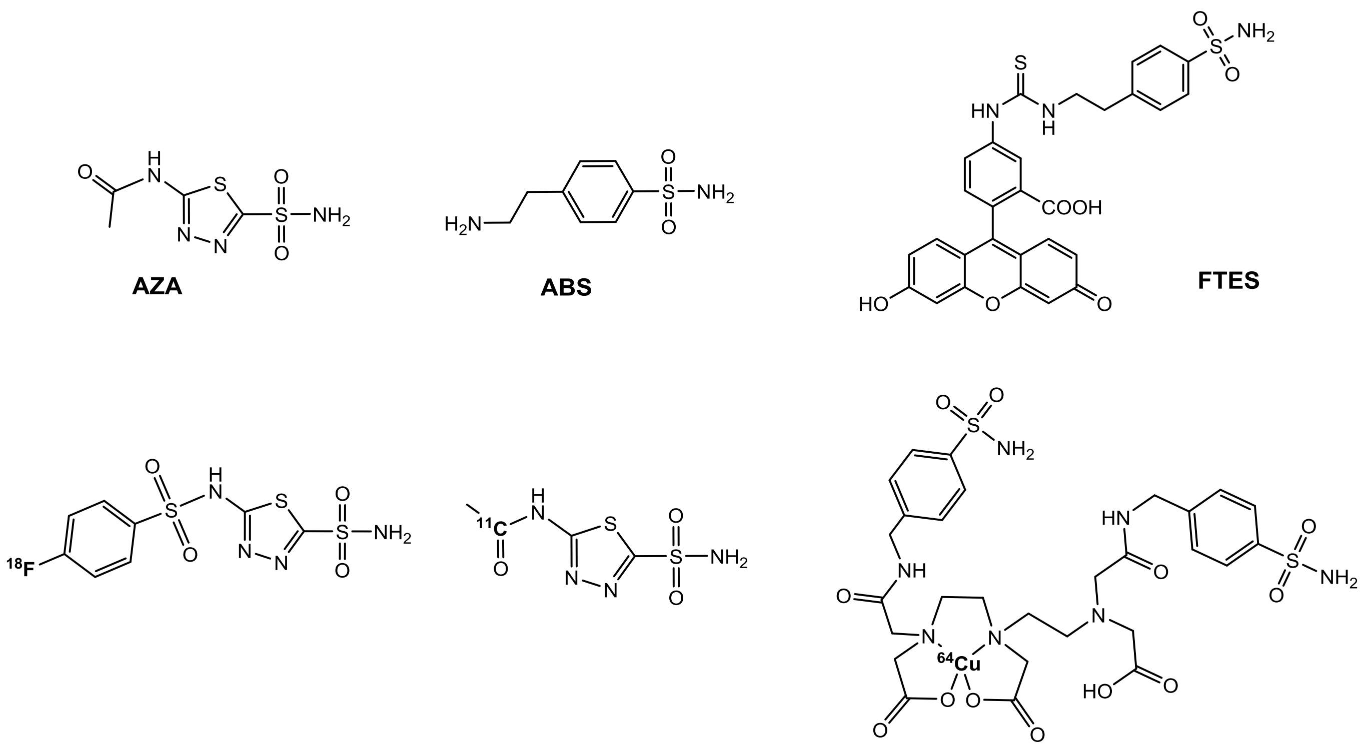

Figure 1: Schematic representations of: acetazolamide (**AZA**), 4-(2-aminoethyl) benzenesulfonamide (**ABS**), fluorescein-thioureido-ethyl-sulfonamide (**FTES**) and of three known reagents radiolabelled with [18-F], [11-C] or [64-Cu] for PET imaging *in vivo*.

Derivatives of **AZA** and **ABS** have been investigated as bifunctional chelators for imaging. However only very few examples of these derivatives labelled with radioisotopes have been reported thus far (Figure 1). [12] Preliminary radiolabelling studies with 2-[^{18}F]-3,5,6-trifluor-3'-sulphamoylbenzanilide have demonstrated good correlation between ^{18}F retention and the level of the CA IX expression *in vitro* and favourable tumour to muscle uptake ratios for CA IX expressing HT-29 xenograft models *in vivo*.[13] Bifunctional compounds of these sulfonamides have been developed to incorporate a fluorescent tag in the form of fluorescein [14] to enable *in vitro* fluorescence imaging. Upon incorporation of a linker such as EDTA or DTPA for the binding of copper (Figure 1) interest could be focused on PET radiolabelling studies.[15] Metal-complexed sulfonamides are particularly interesting as in some instances the binding affinity of the conjugate has been shown to exceed that of the parent sulfonamide due to the simultaneous binding of the sulfonamide to the catalytic zinc centre within the CA active site, and of the metal ion to a histidine residue critical to the catalytic cycle of CO_2 processing.[16,17,18] The compound fluorescein-thioureido-ethyl-sulphanilamide

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(**FTES**) has already been shown to stain selectively cells which up-regulate the CA IX isozyme. In silico docking studies of the enzyme suggested that hydrogen bonding accounts for the increased affinity for CA IX over CA II with a guanidine residue specific to CA IX participating in hydrogen bonding with the carbonyl oxygen of the fluorescein tail of the inhibitor.[8] Recently, the Florence group and collaborators also reported on the organometallic [(Cp-R)M$(CO)_3$] (M=Re or 99m-Tc) arylsulfonamide conjugates which were found selective targeting of human Carbonic Anhydrase IX. [8c] This paper reports on the synthesis and characterisation of new conjugates of arylsulfonamides (grouped under three different ligand classes: thiosemicarbazonate-, amidothiolate- and porphyrin-based) and their corresponding metal complexes, *i.e.* complexes of technetium, rhenium, gallium, indium copper and zinc in their non-radioactive or radioactive form (for ^{64}Cu and ^{99m}Tc). Cellular imaging tests were also performed on three (fluorescent) porphyrin ligands and one indium porphyrin complex, aiming to shed light into the cell inclusion of these conjugates and explore the effect of these sulfonamide derivatives on CA-mediated mechanisms. Their capacity to inhibit a series of carbonic anhydrase isozymes were performed via CA enzyme inhibition assays: the observed binding constants were used to assess potential candidates for use as SPECT or PET imaging agents for tumour hypoxia.

Results and Discussion

Arylsulfonamides conjugated with 64-copper(II) or zinc(II) bis(thiosemicarbazonate) complexes

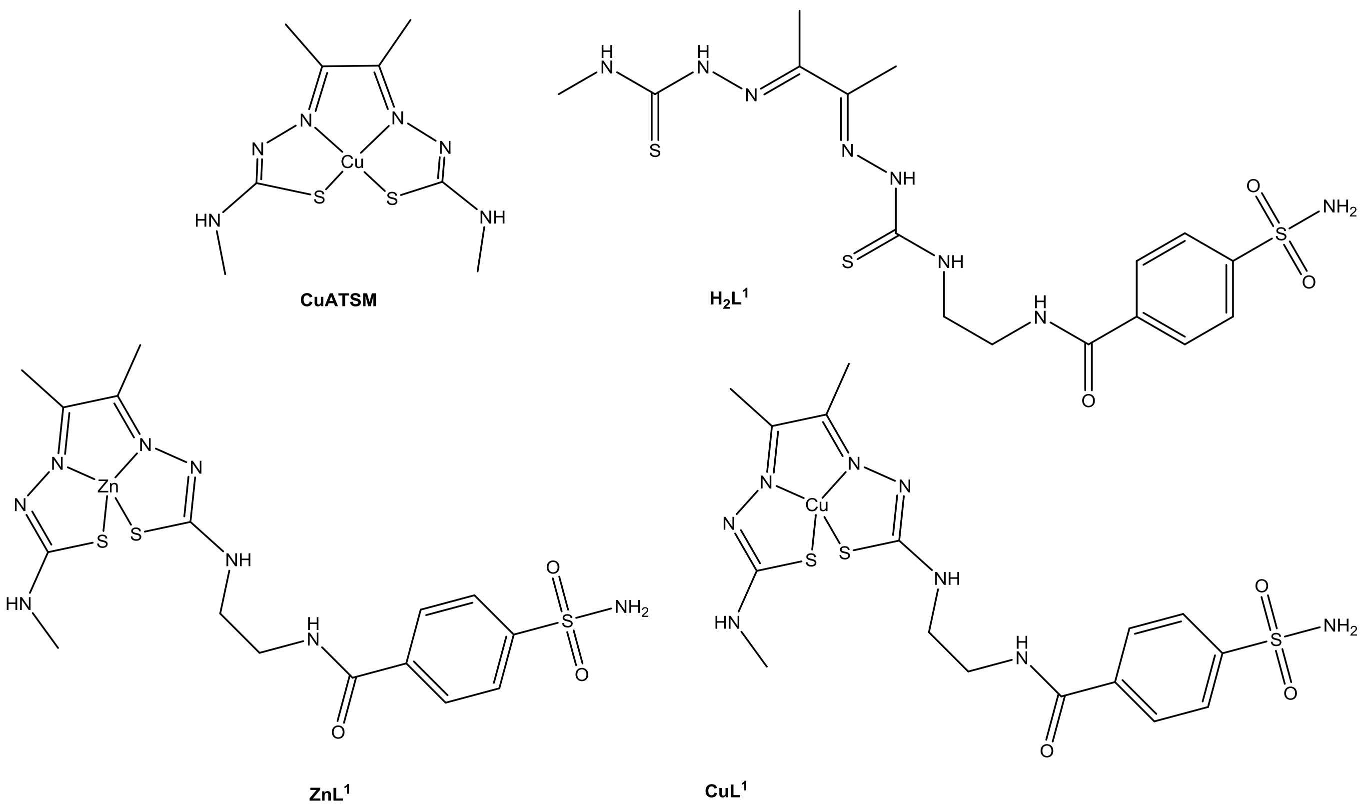


Figure 2: Schematic representations of of CuATSM and the new conjugates emerging from the H_2ATSM/en precursor, H_2**L¹**, Zn**L¹** and Cu**L¹**

The complex diacetyl-*bis*(N^4-methylthiosemicarbazonato)copper(II),CuATSM, Figure 2 has been shown to be selective for hypoxic tissue in imaging studies, where the PET radioisotopes ^{60}Cu, ^{61}Cu or ^{64}Cu were used,[19,20,21] and, as such it has been used clinically for the detection of hypoxia in cancer.[22,23] It has been shown[24] that Cu(II) bis(thiosemicarbazonates) are stable *in vitro* in serum and can also be used to radiolabel bombesin at room temperature without compromising the biological targeting capabilities of the protein. Here, analogous bis(thiosemicarbazide) system was used to form new conjugates with arylsulfonamides (see Figure 3 and Experimental Section). The possible impact on enzyme inhibition of the coordination of zinc or copper to the bis(thiosemicarbazone) ligand conjugate was explored. Synthesis of the *bis*(thiosemicarbazone)-sulfonamide $H_2\mathbf{L^1}$ was accomplished in an 82% yield *via* the carbodiimide-mediated coupling of 4-sulfamoylbenzoic acid with the previously described precursor H_2ATSM/en.[20] The complexes Zn$\mathbf{L^1}$ and Cu$\mathbf{L^1}$ were produced in yields of 81% and 44% respectively by room temperature reaction of $H_2\mathbf{L^1}$ in methanol solution with zinc(II) acetate and copper(II) acetate respectively. ^{64}Cu$\mathbf{L^1}$ was prepared in excellent radiochemical incorporation (> 95%) by reaction of a DMSO solution of $H_2\mathbf{L^1}$ with ^{64}Cu(II) in aqueous acetate buffer.

Arylsulfonamides conjugated to 99m-technetium(V) or 'cold' rhenium(V) complexes

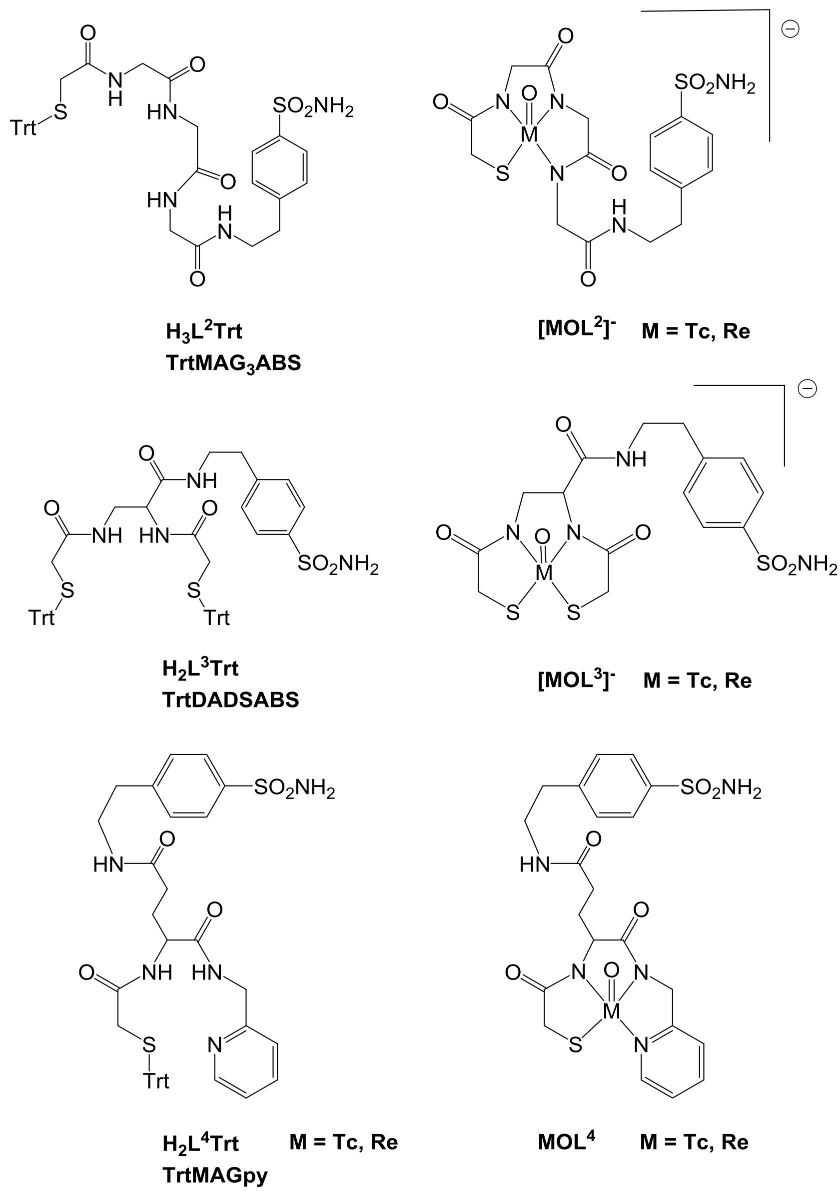
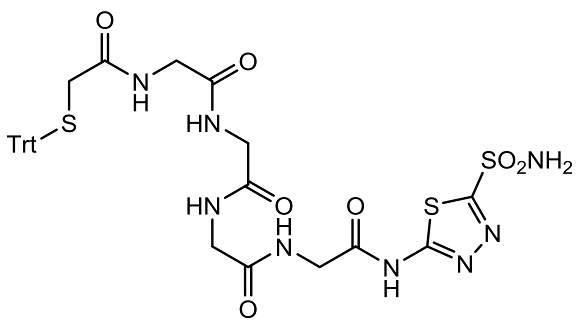


Figure 3: Schematic representations of new protected arylsulfonamide conjugated ligands and complexes with Re and ^{99m}Tc.

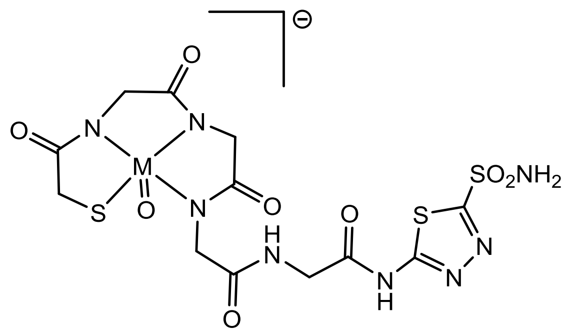
In the case of Tc and Re we used the known MAG_3, DADS and MAGpy ligands (see Figure 3) to study the effect of overall charge and ligand structures on CA enzyme inhibition. The MAG_3 and DADS ligands have been used extensively with 99m-Tc and the complexes are rapidly and specifically excreted from the body *via* the kidneys[25,26]**,** a desirable attribute for radiotracers. The technetium(V) or rhenium(V) complexes of MAG_3 and DADS share the same charge (-1) but have structural differences, while the neutral complex of MAGPy is different in both charge and structure.[25,27] For the sulfonamide conjugates, high efficiency of

CAIX binding requires a favourable steric fit into the inhibitor binding site of the CAIX enzyme[28] and secondary interactions such as H-bonding may be important.

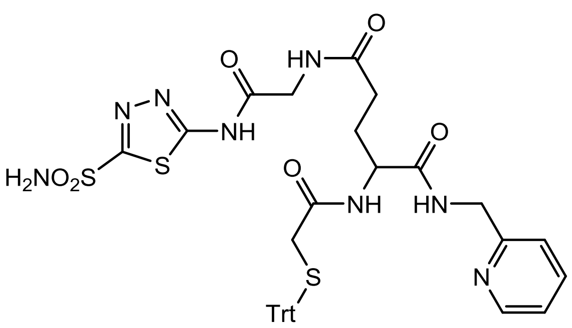
In this study, three known ligands for Tc and Re (MAG_3, DADS and MAGPy), all bearing a pendant carboxylic acid group were synthesised and are reported. **ABS** (Figure 1) and glycinated acetazolamide (**Gly-AZA**) containing a reactive amino group available for linking to a sulfonamide were first synthesised. These proligands (H_3L^2Trt, H_2L^3Trt and H_2L^4Trt where Trt = trityl, Figures 3 and 4) were obtained *via* standard amide formation using a coupling agent (EDC or BOP, Experimental section). These conjugates were then successfully labelled with ^{99m}Tc as the Tc(V) mono-oxo dervatives. Because technetium and rhenium are in the same group of the periodic table and share similar chemical and biological properties[29], the bioassay results of the rhenium and technetium complexes are directly comparable. Therefore, due to the need to use a 'cold' HPLC confirmation method for the ^{99m}Tc complexes and to proceed to further studies on CAs inhibtion studies the isostructural rhenium complexes were also synthesised and fully characterised, as described in Experimental section.



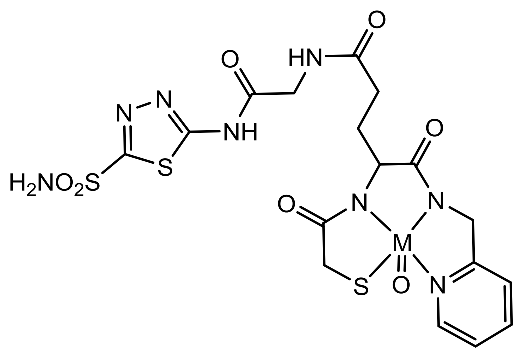
H_3L^5trt



$[MOL^5]^-$ M = Tc, Re



H_2L^6trt

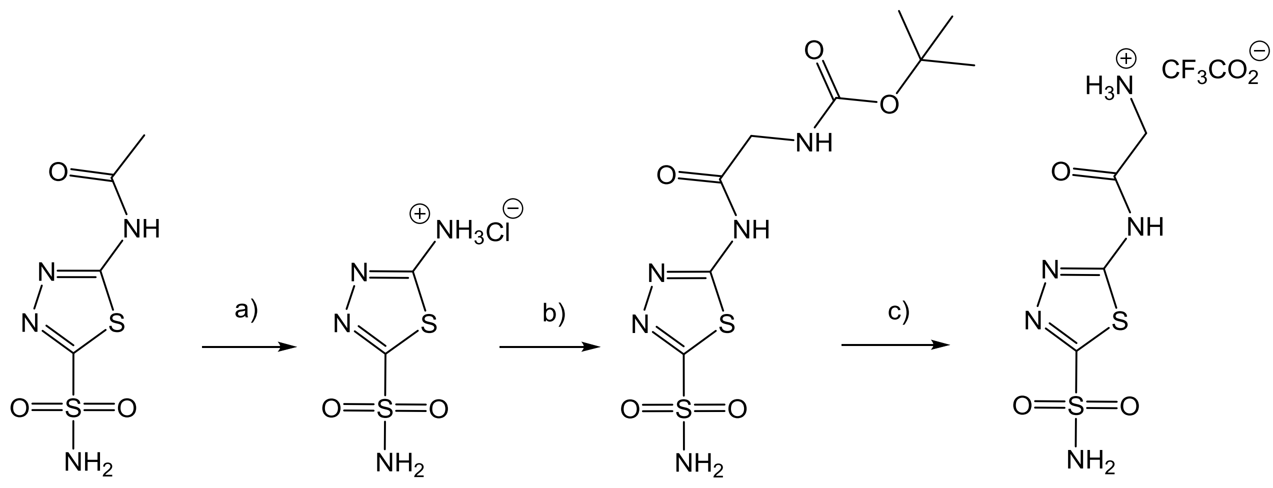


MOL^6 M = Tc, Re

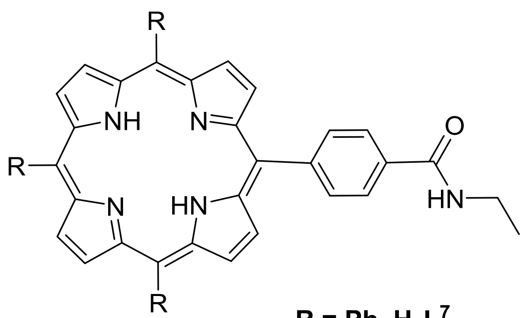
Figure 4: Schematic representations of the new protected acetazolamide conjugated ligands and complexes with Re(V) and ^{99m}Tc(V)

Arylsulfonamide conjugates with porphyrins and metalloporphyrins

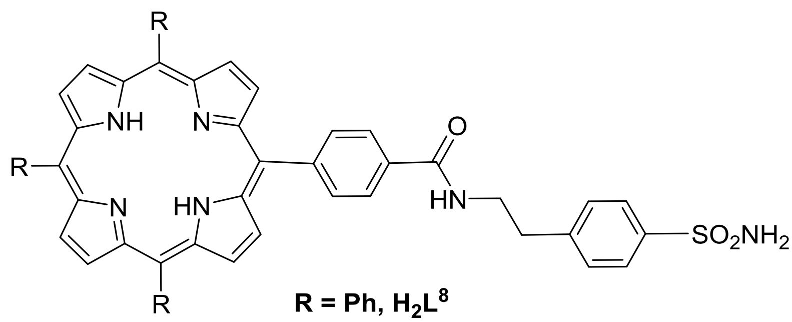
To explore if metalloporphyrins conjugated to sulfonamides would also target CAIX a range of porphyrins (Figure 5) conjugated to ABS or GlyAZA were synthesised together with their indium(III) and gallium(III) complexes. These were of interest as they provide the possibility of using the intrinsic fluorescence of the porphyrins to study cellular uptake mechanisms and tissue distributions *in vivo.* The peripheral porphyrin substituents were selected deliberately aiming to vary the overall charge and water solubility. The amide and sulfonamide monofunctionalised tetraarylporphyrin or tripyridylarylporphyrin ligands ($H_2\mathbf{L^7}$ through to $H_2\mathbf{L^{13}}$) were synthesised from the carboxylate using standard coupling reactions with the appropriate amino sulfonamide. Whilst ABS is commercially available as the free amine, the structure of AZA is not suitable for direct amide coupling. Therefore, as shown briefly in Scheme 1, an amine functionalised sulfonamide was coupled *via* an amide linker to a carboxyphenyl porphyrin precursor, using either the NHS activated ester or using BOP as a coupling agent with the porphyrin precursor of choice, which incorporates a carboxylate group. To achieve a reliable nucleophilic amine source a Boc-glycine linker was introduced according to Scheme 1. Deprotection of the Boc group with TFA yielded a final functionalised AZA derivative suitable for coupling to the porphyrin scaffolds according to procedures described in Scheme 1, Experimental section and ESI. Figure 5 gives the schematic representation of the new free base porphyrin derivatives synthesised.



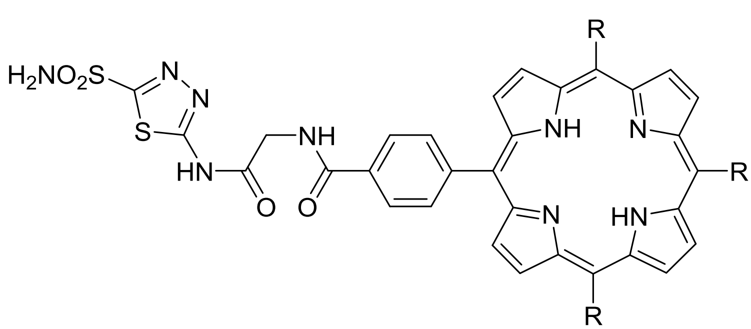
Scheme 1: Schematic synthesis of derivatised acetazolamide for the aryl carboxylate-functionalised porphyrin coupling: a) conc. HCl, EtOH, reflux 3 h; b) Boc-glycine, NEt_3, ^{i}Butyl chloroformate, MeCN, -10 °C to rt. 12 h; c) TFA, rt. 3 h.



R = Ph, H_2L^7



R = Ph, H_2L^8
4-$O_3SC_6H_4^-$, H_2L^9
$MeNC_5H_4^+$, H_2L^{10}



R = Ph, H_2L^{11}
4-$O_3SC_6H_4^-$, H_2L^{12}
$MeNC_5H_4^+$, H_2L^{13}

Figure 5: Structural representations of the free base porphyrin derivatives investigated.

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Molecular structure of H_2L^9 by synchrotron X-ray diffraction

Crystals of trisulfonated porphyrin **H_2L^9** were grown by the slow infusion of acetone into an aqueous THF solution (1:1 *v/v* THF:water) of the compound. The crystals produced extremely weak but promising X-ray diffraction patterns only when a synchrotron X-ray source was used (SRS Daresbury, Station 9.8). The small crystal size (0.02 x0.02x0.35 mm), the large size of the unit cell and asymmetric unit, the weak scattering of the atoms involved and the extensive disorder of solvating water molecules made solving and refining this data set extremely challenging. The molecular structure is shown in Figure 6 (CCDC 1028969). The sulfonamide bond lengths and angles are all in accordance with published X-ray crystal structures for aromatic sulfonamide structures[30]. Though the data set was extremely weak, a solution was found and the structure determination allowed the confirmation of the connectivity and thus the identity of this compound, in close agreement with ^{1}H and ^{13}C NMR, and ESI-MS data, and thus the formation of the desired functionalised porphyrin complex. The short amide C-N bond (C(45)-N(5) 1.26 Å) found in H_2**L^9** is again indicative of significant delocalization over the amide group, and extensive disorder in the sulfonamide arm necessitated modeling over several sites during refinement. Despite this, the overall conectivity is clear and shed light into the molecular strucuture of the first water solubile porphyrin-sulfonamide derivative. The crystal packing is described by a series of H-bonding interactions (Figure S1 in ESI) which network between solvent water molecules, sulfonate groups and sulfonamide amine groups, with H-bonding distances ranging between ca. 2.15 Å (between the sulfonamide amine nitrogen and water molecule) for (N(6)-O(13)) and ca. 2.77 Å (between a sulfonate oxygen and water molecule) for (O(8)-O(14)). The intermolecular interactions observed include some weak aromatic interactions (with short range interactions of ca. 2.75 Å) whereby π-π stacking takes place via the T-shape stacking of phenyl rings on adjacent porphyrins (Figure S2 in ESI).

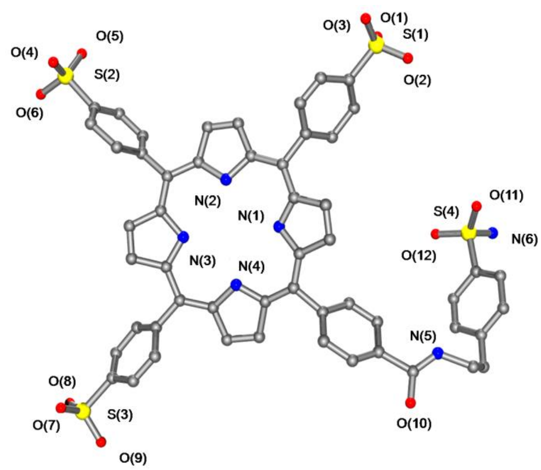

Figure 6: Ball and stick representation of the molecular structure of **H_2L^9**

CAIX Inhibition Assays

Enzyme inhibition data was determined, for all those compounds which could be isolated in satisfactory yields and which showed aqueous solubility, for physiologically dominant hCA I and II and cancer-associated hCA IX and XII by assaying the CA catalysed hydration of CO_2[31] and the data obtained is summarised in Table 1. The entry numbers **1-20** used in the following text refer to those listed in Table 1.

Table 1: Enzyme inhibition studies (binding affinity data Ki (nM)) for sulfonamide series with calculated selectivity ratios.
ABS = arenebenzylsulfonamide, AZA = actetazolamide, FTES = fluoresceinthioureidoethylsulfonamide. ***Errors in the range of ± 5-10% of the reported value, from three different determinations. CAI and CAII are Human (cloned) isozymes. CAIX is the catalytic domain of the human, cloned isozyme. CAXII is the catalytic domain of the human recombinant isozyme, CO_2 hydrase assay method.

No	Compound	K_i (nM)				Selectivity ratios		
		CAI	CAII	CAIX	CAXII	CAI/CAIX	CAII/CAIX	CAXII/CAIX
1	**H_2L^1**	335	470	62	73	5.4	7.5	0.9
2	**ZnL^1**	11.4	9.3	28	6.8	0.4	0.33	0.2
3	**CuL^1**	33.1	9.0	9.0	6.1	3.6	1.0	0.7
4	**$[ReL^2]^-$**	432	1180	105	87	4.1	11.2	0.8
5	**$[ReL^3]^-$**	1030	1110	89	88	11.6	12.5	1.0
6	**$[ReL^5]^-$**	1020	1190	1270	85	0.8	0.9	0.1
7	**H_2L^7**	78	46	82	84	1.0	0.5	1.0
8	**$InClL^7$**	91	111	87	80	1.0	15.1	1.0
9	**H_2L^8**	1080	818	54	46	20.0	15.2	1.0
10	**$GaClL^8$**	442	841	38	71	11.6	22.1	1.9
11	**$InClL^8$**	126	46	68	72	1.9	0.7	1.0
12	**H_2L^9**	2650	5750	435	720	6.1	13.2	1.7
13	**$InClL^9$**	442	841	38	71	11.6	22.1	1.9
14	**H_2L^{10}**	451	770	49	66	9.2	15.7	1.3
15	**H_2L^{11}**	820	651	52	73	15.8	12.5	1.4
16	**$GaClL^{11}$**	433	84	59	42	7.3	1.4	0.7
17	**$InClL^{11}$**	391	39	86	35	4.6	1.0	1.0
18	**H_2L^{12}**	2970	3986	518	792	5.7	7.7	1.5
19	**H_2L^{13}**	655	520	39	45	16.8	13.3	1.1
20	**$InClL^{13}$**	77	81	79	97	1.0	1.0	1.2
Control	**ABS**	2100	160	33	3.2	63.6	4.9	0.1
Reference 14b	**AZA**	250	12	25	5.7	10	0.5	0.02
Control	**FTES**	1300	45	24	-	54.2	1.9	-

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In the cases of the bis(thiosemicarbazonates) studied (i.e. with Compound entries with No's **1-3** in Table 2), the free ligand and the metal complexes all show low K_i values for CaIX and CaXII inhibition, that for the Cu complex being almost equivalent to ABS (given at the bottom of the table, and used as a control) itself. However, the discrimination between CaIX and the other isoforms is poor. The anionic Re complexes under entries **4-6** all appear to be active, and promising substrates to be used in this context however they show selectivity differences that are difficult to rationalise on the basis of structure or targeting group. The rhenium complex under entry **5** with a pendant sulfonamide has the best overall performance in this assay. Intriguingly, the free base porphyrin $H_2\mathbf{L}^{\mathbf{7}}$ and its corresponding indium complex, which possess no sulfonamide functionality, both show reasonably strong binding affinities for the CA enzymes but in both cases show poor selectivity for CA IX and XII over both CA I and II. Introduction of the sulfonamide entities ABS (to give compound H_2L^8) increases the binding affinity for the CA IX and XII enzymes and dramatically increases the selectivity for these enzymes over the non-target CA I and II. In terms of CAIX inhibition with the porphyrins and metal complexes it is reassuring that the porphyrin derivative (see entry **7** in Table 1) with no sulfonamide shows no selectivity for CAIX whereas the free base compound shown under entry **9** (having one aryl replaced by a pendant arylsulfonamide, $H_2\mathbf{L}^{\mathbf{8}}$) shows strong discrimination between CAIX and other isoforms and is one of the most effective compounds of those screened in this study. However the introduction of either indium or gallium to give the metal complexes results in a loss in selectivity particularly with the indium complexes. Disappointingly, from the perspective of using these compounds for PET or SPECT imaging after incorporation of a metal-halide centre (e.g. In(III)-Cl or Ga(III)-Cl) and somewhat surprisingly, this generally appears to reduce the selectivity for CAIX over the other isoforms significantly. Free-base sulfonated porphyrin H_2L^9 (entry **12**) and its corresponding In-Cl complex (entry **13**) provide an exception to this with the In complex showing both stronger inhibition and higher selectivity. As with the metal complexes mentioned above there is no obvious correlation of the observed binding/selectivity characteristics with structure. It is likely that subtle factors involving secondary interactions within the binding pocket are dictating the overall inhibition patterns. Based on these results complexes under entries **5** and **13** are under current investigations in our laboratories, and are being taken forward for *in vitro* and *in vivo* studies using the ^{99m}Tc and ^{111}In labelled variants respectively. For the AZA-derivatised metallo-porphyrin compounds ($H_2\mathbf{L}^{\mathbf{11}}$, $H_2\mathbf{L}^{\mathbf{12}}$ and $H_2\mathbf{L}^{\mathbf{13}}$) we observed a lower binding affinity to the CA XI and XII enzymes, whilst for the ABS metallo-porphyrin species we see an increased binding affinity for the CA XII enzyme. The shift from H_2TPP to the pyridinium-functionalised porphyrin species results in a reduction in the selectivity for CA IX and XII over both CA I and II but the binding affinity to CA IX and CA XII is still favourable for both AZA- (H_2L^{13}) and ABS- (H_2L^{10}) functionalised species.

Introduction of indium to the tetrapyrrole ring results in complete lack of selectivity towards CA IX and XII with a diminished binding affinity to both enzymes. This suggests that either the free base porphyrin provides additional non-covalent bonding interactions that enhance the overall binding affinity and selectivity towards the CA IX and CA XII enzymes or that the ubiquitous CA enzymes I and II provide additional interactions with the metallo porphyrins which enhance the binding interactions with these enzymes more so than with CA IX and CA XII. Shifting from H_2TPP to the sulfonate species ($H_2\mathbf{L}^{\mathbf{9}}$) still shows a strong affinity for CA IX and XII over both CA I and II but the binding affinity for all the enzymes is dramatically reduced suggesting that the sulfonate groups act to inhibit binding, conversely introduction of the metal ion dramatically increases the binding affinity and selectivity for CA IX and XII over CA I and II. In general, all of the porphyrin species show a uniform medium strength binding affinity to CA IX and CA XII enzymes. All sulfonamide species show an enhanced selectivity for CA IX and CA XII over CA I and CA II in comparison to the porphyrin species alone indicating that the presence of the sulfonamide porphyrin conjugate has a positive effect towards improving selectivity. The selectivity ratios and binding affinities however are much poorer than the control sulfonamides **ABS** and **AZA** on their own and the fluorescein-thioureido-ethyl-sulphanilamide (**FTES**) ligand suggesting that the intrinsic affinity that the porphyrin group has for the CA enzymes prevents the sulfonamide groups from binding preferentially to the CA IX and CA XII enzyme isoforms. Several of the compounds do however show particularly favourable binding affinities such as the free base porphyrins H_2L^8 and the sulfonate variant H_2L^9.

Docking studies, in line with those already reported by Supuran et al. will be carried out in future investigations to elucidate in full the structure activity relationships and the CA inhibition mechanism.[8b]

Confocal fluorescence imaging tests

To assess the binding potential of the sulfonamide groups to the CA IX receptors, confocal fluorescence studies were performed with compounds $H_2\mathbf{L^8}$, $H_2\mathbf{L^9}$ and $H_2\mathbf{L^{10}}$ on the HCT 116 colon carcinoma cell line. The HCT 116 line has been transfected to over-express the CA IX enzyme (without the need for hypoxic culturing) and denoted CAIX positive. The empty-vector cell line, i.e. showing no expression of the CA IX enzyme, was denoted CAIX negative and was used as a control.

Cells lines were cultured according to known protocols[42] and as described below. Cells were seeded on to glass cover slips and left to adhere for 12 h overnight. Cells were incubated with free-base porphyrin compounds $H\mathbf{L^8}$, $H\mathbf{L^9}$, $H\mathbf{L^{10}}$ at 10 μM concentrations for 2 h at 37 ^oC. After 2 h, the cells were washed three times with PBS and confocal images recorded using a Zeiss LSM 510 META microscope, irradiating at 405 nm with emission collected above 565 nm. In each case a sample of 5 images were recorded. Images shown in Figures 7 and 8 represent a fair sample of the recorded images. The two most notable observations are: (a) the different porphyrin species investigated, whereby R = Ph ($H\mathbf{L^8}$), 4-$O_3SC_6H_4$ ($H\mathbf{L^9}$) and $MeNC_5H_4^+$ ($H\mathbf{L^{10}}$) show distinct cellular distributions; and (b) there is no distinguishable difference between the uptake in the positive CA IX expressing cell line and the empty vector cell line. Cellular imaging suggests that the uptake and localisation is strongly driven by the porphyrin unit and not by the sulfonamide groups. The H_2TPP-type derivative $H_2\mathbf{L^8}$ shows strong cytosol uptake with no nuclear staining.

The sulfonate species $H_2\mathbf{L^9}$ shows only very weak internalisation, as may be expected from the negative charge associated with the conjugate, whilst the pyridinium analogue $H_2\mathbf{L^{10}}$ shows strong nuclear uptake in both cell lines. For compound H_2L^{10}, nuclear uptake was confirmed by colocalisation with the nuclear stain DAPI (as shown in Figure 9). The nuclear uptake of the pyridinium species mirrors the nuclear localisation observed by confocal fluorescence in the case of a positively charged tetra(methylquinolinium) porphyrin identified as a G-quadruplex interactive agent.[43] In line with observations from the binding affinities measurements, it appears that the porphyrin unit dominate the cellular uptake of the conjugate. The porphyrin sulfonamides still had some affinity for the CA enzymes. However, the precise discrimination in terms of specific selectivity for the tumour expressing CA IX (and CA XII) enzyme(s) with respect to the other isoforms is not supported by this study.

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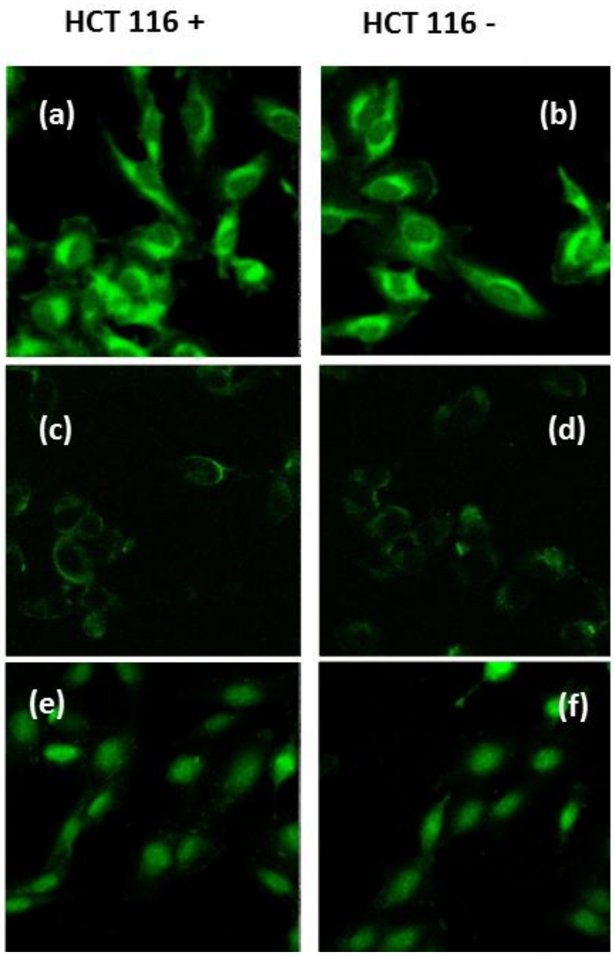

Figure 7: Confocal fluorescence imaging: cellular uptake images of H2L8 (a) and (b); H2L9 (c) and (d); H2L10 (e) and (f) in HCT 116 (CA IX positive, denoted HCT 116+ and CA IX negative denoted HCT 116−) cell lines. Typical bright-field images for the confocal fluorescence micrographs are provided in ESI†. Conditions are described in the text.

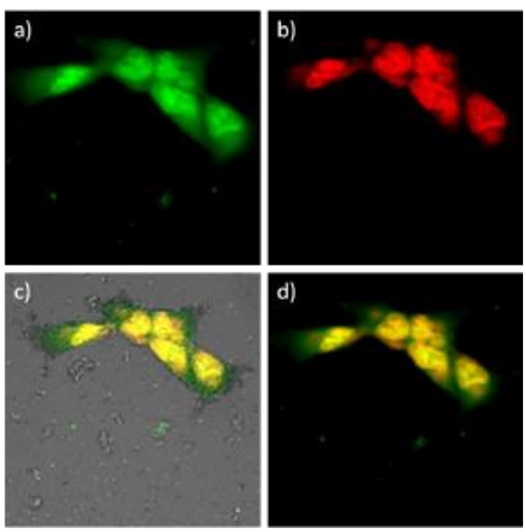

Figure 8: Confocal fluorescence imaging showing the cellular uptake at 37 °C: a) H_2L_{10} (λex = 405 nm, λem > 565 nm); b) DAPI nuclear stain (λex = 405 nm, λem = 465-535 nm); c) overlay showing the simultaneous imaging with H_2L_{10} and DAPI nuclear stain; d) overlay of H_2L_{10}, DAPI nuclear stain and brightfield image. Scalebar = micrograph width= 150 µm.

Radiolabelling tests for CAIX binding by the porphyrin $^{111}In(Cl)L^{9}$

Whilst the porphyrin species do not show the same favourable differentials between CA IX and XII over both CA I and II as the small sulfonamide groups on their own, the medium strength binding affinities and reasonable selectivity ratios are not discouraging for all of the derivatives. Of particular interest is the sulfonate derivative which when present as the metal complex **InClL⁹** shows between 5-10 fold increased selectivity for CAIX over CA I and CAII. Whilst the **H_2TPP** and pyridinium species show by confocal fluorescence rapid cellular uptake by a mechanism independent of CA IX expression, the sulfonate species displayed less cellular association. This is probably a result of the negative charge on the porphyrin. This may make it more suitable for CA binding in the same manner as the fluorescein derivative. **FTES** shows little cellular uptake as a result of its negatively charged free acid group but binds selectively to the membrane bound CA IX enzymes. The selectivity of the fluorescein derivative is only observed by confocal fluorescence after incubation for 24 h with a clear delineation of the cell membrane in CA IX expressing cells. As such uptake studies with the sulfonate compounds were repeated with 24 h incubation at 37 ^oC for **H_2L^9** and **$InClL^9$**. After 24 h it is observed that internalisation is now strongly evident with uniform uptake throughout the cytoplasm. No apparent difference between the HCT 116 CA IX positive and negative cell lines is observed. However it was noted that the cell lines after 24 h incubation showed clear signs of cell death, and uptake mechanisms independent of CA IX expression cannot be ruled out. Further studies will be required to measure the toxicity of these compounds. Incubation at concentrations orders of magnitude lower to reduce toxicity results in much weaker fluorescence and subsequently cellular uptake is not discernible from background autofluorescence. Whilst the porphyrins are fluorescent their quantum yields are still very weak (0.06 and 0.03 for **H_2L^9** and its corresponding indium derivative **$InClL^9$**, respectively) when compared to fluorescein (0.95) and so low concentrations (nanomolar range) of the compounds cannot be efficiently detected by fluorescence. To investigate further the porphyrins at lower concentrations radiolabelling studies have been performed.

Compound **H_2L^9** was radiolabelled using an adapted method with respect to previously published conditions[42] with $^{111}InCl_3$ and in vitro uptake studies carried out in the same two cell lines as used for confocal fluorescence tests. After 1, 3, 6 and 18 h incubation (37 ^oC) of compound H_2L^9 with both the CA IX positive and CA IX negative cell lines the cells were washed with PBS (to remove activity in the supernatant), 1M HCl (to remove activity associated with the cell membrane) and 1M NaOH (to lyse the cells of internalised activity) sequentially, and the supernatant, membrane associated activity and internalised associated activity isolated respectively and measured separately. Cell percentage uptake is given as the amount of activity internalised i.e. the activity in the 1 M NaOH wash with respect to the total activity and the amount of activity associated with the membrane i.e. the activity in the 1M HCl wash with respect to the total activity. The uptake data suggests low uptake in line with the fluorescence images. Identical weak membrane associated activity is measured for both cell lines suggesting that no sulfonamide binding occurs in the CA IX positive cell line (Figure 9).

Cell uptake is observed which may be mediated by sulfonamide binding but is more likely to arise from the intrinsic uptake of the porphyrin conjugate. A higher percentage of radioactivity is associated with the CA IX positive cell line, which might suggest some positive sulfonamide response, but there is potential for variation in the amount of protein in the different cell population, despite the same initial cell concentration. Detailed protein assays are beyond the scope of this work and were not completed so the difference in internalised activity as a result of difference in cell population cannot be immediately ignored. Comparable changes in cell uptake in both cell lines over the time course are observed for the total cell associated activity with a difference in associated activity over 18 h of only 1.5 %. This again suggests that there is no CA mediated mechanism of cell inclusion of these porphyrin conjugates.

A closer inspection of the CA binding data (Table 1) suggested that the sulfonamide species **$InClL^9$** has an increased binding affinity of 5-10 fold in a CA IX expressing cell line based on binding affinities alone. This should lead to increased membrane and potentially increased internalised associated activity. On the basis of radiolabelling studies no such increase in uptake or binding is observed, which is in accord with the confocal fluorescent studies and suggests strongly that uptake and/or binding is not receptor-mediated via initial carbonic anhydrase recognition. Furthermore, the anionic sulfonamides show no apparent recognition for the CA IX receptors and the weak uptake is most probably driven by the intrinsic accumulation of the porphyrin entity. Given the rapid and strong cellular uptake of the cationic and neutral porphyrin species it was felt unlikely that they

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would confer any better differential, with their cellular uptake dominated purely by the porphyrin entity and by a pathway independent of sulfonamide binding to CA IX enzymes.

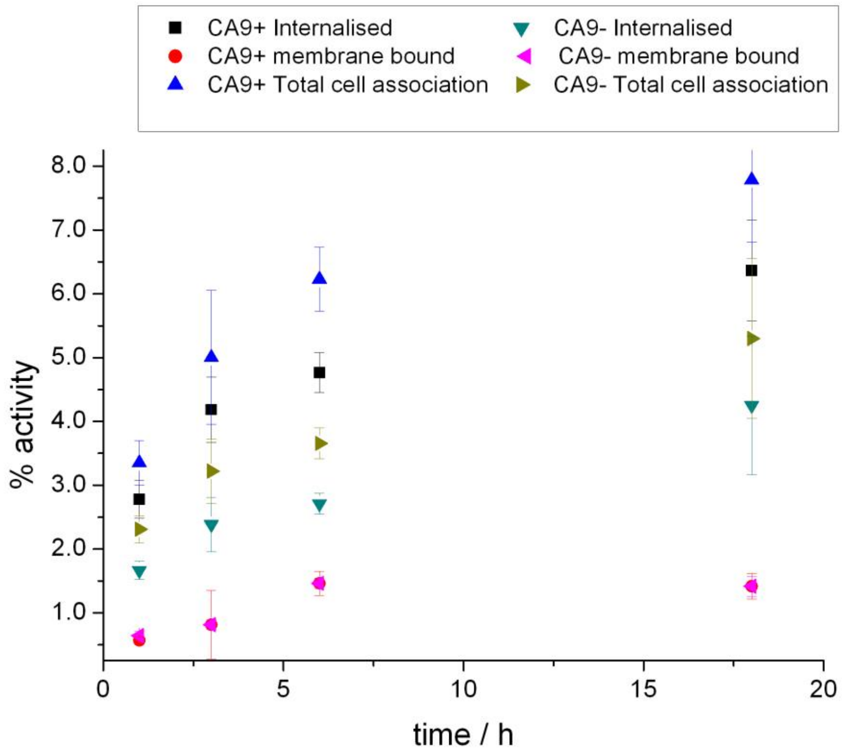

Figure 9: Uptake of [111-In]-labelled $\mathbf{H_2L^9}$ species in HCT116 (CA IX positive) and HCT116 (CA IX negative) cell lines showing % activity associated with the cell membrane and internalised for each cell line. Experiments were carried out in triplicate and error bars indicate one standard deviation of the mean**.**

Conclusions

A range of new sulfonamide conjugated metal complexes involving bis(thiosemicarbazonate), amidothiolate and porphyrin ligands have been prepared with a view to developing PET and SPECT agents based on metallic radionuclides to target CAIX expression *in vivo*. These compounds were all fully characterised and successful, radiolabelling protocols resulting in a high radiochemical incorporation were established for the 64-Copper and 99m-Technetium complexes. Those complexes with solubility and stability in an aqueous environment suitable for the CA enzyme inhibition assay were screened for activity, by testing their ability to inhibit CAIX and other isoforms of the enzyme *in vitro*. A series of sulfonamide porphyrin conjugates has been synthesised and fully characterised. Water soluble analogues have been developed for greater biocompatibility with no sign of acid hydrolysis of the amide bonds under sulfonating conditions and no sign of alkylation of the thiadiazole ring under methylating conditions. A library of sulfonamide derivatives has been studied for their binding affinities to specific CA receptors to establish their affinity for the poor prognostic CA IX and CA XII enzymes. Our findings for the rhenium derivatives are comparable with those recently reported for organometallic [(Cp-R)M(CO)$_3$] (M=Re or 99m-Tc) Arylsulfonamide conjugates investigated for the selective targeting of human Carbonic Anhydrase IX. [8c] In general the porphyrin sulfonamide conjugates displayed positive selectivity for the CA IX and CA XII enzymes though metal insertion into the porphyrin resulted in loss of selectivity and reduced binding affinity. Conversely the sulfonate porphyrins showed the reverse effect showing improved binding and increased selectivity for CA IX and CA XII on indium complexation. Fluorescent studies in a CA IX positive and CA IX negative cell line indicate (after 2 h and 24 h incubation at 37 ^{o}C) that there is no obvious difference in the cellular binding or uptake between the two cell lines for the sulfonyl porphyrin species. A strong structural correlation with cellular uptake is observed with the neutral tetraphenyl porphyrins showing strong cytoplasm uptake, the cationic porphyrins showing nuclear accumulation whilst the sulfonyl porphyrins display only weak cell uptake. Radiolabelling studies with an indium(III)- labelled sulfonated **ABS** porphyrin identified identical weak membrane binding for the CA IX positive and CA IX negative cell lines and comparable cell internalisation suggesting that the conjugates cellular distribution and uptake was dominated by the porphyrin core. Although these results showed no direct correlations between the structures and physical properties of the complexes and the observed activities, two compounds based on the porphyrin motif were identified as being good candidates to take forward to cellular and *in vivo* investigations.

Experimental Procedures

General: All reagents were purchased from Sigma-Aldrich, Merck Chemicals or Alfa-Aesar and were used as received unless otherwise stated. Na[$^{99m}TcO_4$] was obtained from a $^{99}Mo/^{99m}Tc$ generator (GE Healthcare, UK). H_2ATSM and H_2ATSM/en were prepared according to published methods.[32,33] ESI contains further details on HPLC and radioHPLC methods A-C used and intermediates' syntheses.

Diacetyl(N-4-methylthiosemicarbazone)(N-4-aminoethylamidophenyl-4-sulphamoylthiosemicarbazone), H_2L^1,

H_2ATSM/en (150 mg, 0.51 mmol), 4-sulfamoylbenzoic acid (104 mg, 0.51 mmol), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (119 mg, 0.62 mmol) and hydroxybenzotriazole (84 mg, 0.62 mmol) were placed under N_2 in a schlenk flask and DMF (5 ml) added. The yellow mixture was stirred for 18 h at room temperature and water (50 ml) added generating a yellow precipitate. This was collected by filtration, washed with water and diethylether, and dried in vacuo to provide H_2**L** (197 mg, 0.42 mmol, 82%).

^{1}H NMR (300 MHz, DMSO-d$_6$) δ ppm 10.34 (s, 1H, N*H*N=C), 10.24 (s, 1H, N*H*N=C), 8.88 (t, *J* = 5.3 Hz, 1H, CH$_2$N*H*C=S), 8.60 (t, *J* = 5.3 Hz, 1H, CH$_2$N*H*C=O), 8.39 (q, *J* = 4.40 Hz, 1H, CH$_3$N*H*C=S), 8.00 (d, *J* = 8.50 Hz, 2H, Ar*H*), 7.89 (d, *J* = 8.50 Hz, 2H, Ar*H*), 7.49 (s, 2H, SO$_2$N*H$_2$*), 3.78 (td, *J* = 5.7, 5.3 Hz, 2H, C*H$_2$*NHC=S), 3.54 (td, *J* = 5.7, 5.3 Hz, 2H, C*H$_2$*NHC=O), 3.02 (d, *J* = 4.51 Hz, 2H, C*H$_3$*NHC=S), 2.23 (s, 3H, C*H$_3$*C=N), 2.21 (s, 3H, C*H$_3$*C=N). ^{13}C{^{1}H} NMR (75.5 MHz, DMSO-d$_6$) δ ppm 178.5 (*C*=S), 178.1 (*C*=S), 165.9 (*C*=O), 148.4 (*C*=N), 148.0 (*C*=N), 146.3 (Ar), 137.2 (Ar), 128.0 (Ar), 125.6 (Ar), 43.9 (*C*H$_2$), 38.9 (*C*H$_2$), 31.3 (*C*H$_3$NH), 11.8 (*C*H$_3$C=N), 11.7 (*C*H$_3$C=N). MS ES$^+$: found 495.1, calc. for $[M + Na]^+$ ($C_{16}H_{24}N_8NaO_3S_3$) 495.1; ES$^-$: found 471.1, calc. for $[M - H]^-$ ($C_{16}H_{23}N_8O_3S_3$) 471.1. HPLC R_t = 10.86 min

Diacetyl(N-4-methylthiosemicarbazonato)(N-4-aminoethylamidophenyl-4-sulphamoylthiosemicarbazonato)zinc(II), ZnL^1

H_2L^1 (40 mg, 85 μmol) and $Zn(OAc)_2.2H_2O$ (22 mg, 100 μmol) were stirred together in methanol (5 ml) at room temperature for 2 h forming a bright yellow solution. Water (20 ml) was added and the solution evaporated to remove the methanol. The solution was extracted with ethyl acetate (3 x 10 ml) and the combined organic fractions dried ($MgSO_4$), evaporated and dried under vacuum to give ZnL^1 as a bright yellow solid (37 mg, 69 μmol, 81%).

^{1}H NMR (300 MHz, DMSO-d$_6$) δ ppm 8.72 (t, *J* = 4.5 Hz, 1H, CH$_2$N*H*C=O), 7.97 (d, *J* = 8.5 Hz, 2H, Ar*H*), 7.88 (d, *J* = 8.5 Hz, 2H, Ar*H*), 7.49 (s, 2H, SO$_2$N*H$_2$*), 3.51-3.44 (m, 2H, C*H$_2$*), other C*H$_2$* obscured by water peak, 3.16 (d, *J* = 5.0 Hz, 2H, C*H$_3$*NHC=S), 2.20 (s, 3H, C*H$_3$*C=N), 2.18 (s, 3H, C*H$_3$*C=N). HRMS ES$^+$: found 535.0320, calc. for $[M + H]^+$ ($C_{16}H_{23}N_8O_3S_3Zn$) 535.0347. HPLC R_t = 10.12 min

Diacetyl(N-4-methylthiosemicarbazonato)(N-4-aminoethylamidophenyl-4-sulphamoylthiosemicarbazonato)copper(II), CuL^1

H_2L^1 (40 mg, 85 μmol) and $Cu(OAc)_2.2H_2O$ (22 mg, 100 μmol) were stirred together in methanol (5 ml) at room temperature for 2 h forming a brown solution. Water (20 ml) was added and the solution evaporated to 1/3 volume to produce CuL as a brown precipitate, which was collected by filtration and dried under vacuum (20 mg, 38 μmol, 44 %). HRMS ESI$^+$: found 534.0364, calc. for $[M + H]^+$ ($C_{16}H_{23}N_8O_3S_3Cu$) 534.0351. HPLC R_t = 11.98 min.

Syntheses of technetium and rhenium conjugates

N-(2-oxo-2-((2-oxo-2-((4-sulfamoylphenylethyl)amino)ethyl)amino)ethyl)-2-(tritylthio)acetamide TrtMAG$_3$-ABS, TrtH$_3$L^2

Succinimido-2-((triphenylmethyl)thio)acetate **(1)** was prepared following literature procedures[34, 3]. To a solution of **(1)** (3.44 g, 7.98 mmol) in 1,2-dimethoxyethane (35 mL) and DMF (18 mL) was added a solution of triglycine (1.52, 8.04 mmol) and $NaHCO_3$ (1.38 g) in water (18 mL). The resulting solution was stirred at RT for 3 h and then concentrated *in vacuo* to remove 1,2-dimethoxyethane. Dilution with water (35 mL) and treatment with 50% aqueous citric acid (6 mL) gave a white solid of Trt-MAG$_3$ (**2**), which was recrystallized in ethanol (2.90 g, 71.9 %).

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To a solution of **(2)** (393 mg, 0.777 mmol) in DMF (5 mL) was added ABS (155 mg, 0.774 mmol), EDC (149 mg, 0.777 mmol), HOBT (104 mg, 0.770 mmol) and TEA (24 µL). The reaction was left in ice bath for 1 h and then at RT for 1 day. The solvent was evaporated thereafter. The residue was re-dissolved in ethyl acetate (10 mL) and washed with 10% citric acid, 3% $NaHCO_3$ and saturated brine, 20 mL each. The solids, which precipitated when washed with $NaHCO_3$ and saturated brine, were collected. The organic solution was collected and dried under vacuum. The solutions of the raw product were combined and recrystallized from ethanol/water to give $TrtH_3L^2$ (440 mg, 82.6%).

^{1}H NMR (d_6-DMSO, δ, 300 MHz):2.75 (t, *J*=7.2Hz, 2H), 2.84 (s, 2H), 3.25 (m, 2H), 3.61 (m, 4H), 3.68 (d, *J*=4.9Hz, 2H), 7.21-7.37 (m, 17H), 7.71 (d, *J*=8.3Hz, 2H), 7.92 (t, *J*=5.3Hz, 1H), 8.12 (t, *J*=5.7Hz, 1H), 8.22-8.25 (m, 2H). ^{13}C NMR (d_6-DMSO, δ, 75 MHz): 35.2, 36.4, 42.5, 42.6, 42.8, 42.8, 66.4, 126.1, 127.3, 128.5, 129.5, 129.5, 142.5, 144.0, 144.4, 168.2, 169.1, 169.5, 169.6. ES/MS(+): *m/z*=688.2915 (Calc. *m/z*=688.2264) $[M+H]^+$. HPLC (Method B): R_t=11.88 min.

N,N'-(3-oxo-3-((4-sulfamoylphenylethyl)amino)propane-1,2-diyl)bis(2-(tritylthio)acetamide, TrtDADS-ABS, H_3L^3Trt

Ethyl 2,3-*bis*(triphenylmethylthioacetylamino)propanoate (**3**) was prepared following the literature procedure[35]. To **3** (1.05 g, 1.33 mmol) in a 1:1 mixture of THF and water (80 mL), NaOH (100 mg, 2.5 mmol) was added. The mixture was heated to reflux for 3 h under N_2. The mixture was adjusted to pH=2 by addition of 6 M HCl and was concentrated to 30 mL under reduced pressure. The precipitate that formed was collected by filtration and dried under vacuum to give Trt-DADS (**4**) (825 mg, 84.2%). To a solution of **4** (147 mg, 0.199 mmol) in DMF (5 mL) was added ABS (40.0 mg, 0.200 mmol), EDC (37.8 mg, 0.200 mmol), HOBT (26.7 mg, 0.198 mmol) and TEA (6 µL). The reaction was left in ice bath for 1 h and then at RT overnight. The solvent was evaporated and the residue re-dissolved in ethyl acetate (20 mL). The solution was washed with 10% citric acid, 3% $NaHCO_3$ and saturated brine, 20 mL each. The organic layer was collected and dried under vacuum. The raw product was combined and recrystallized from ethanol/water to give $TrtH_3L^3$ (108 mg, 59.0%).

^{1}H NMR (d_6-DMSO, δ, 300 MHz): 2.70 (m, 2H), 2.74 (s, 2H), 2.80 (s, 2H), 3.11 (m, 2H), 3.21 (m, 2H), 4.11 (m, 1H), 7.19-7.34 (m, 32H), 7.71 (d, *J*=8.4Hz, 2H), 7.86 (t, *J*=5.7Hz, 1H), 8.00-8.07 (m, 2H). ^{13}C NMR (d_6-DMSO, δ, 75 MHz): 35.2, 36.4, 36.5, 40.4, 41.1, 53.3, 66.3, 126.1, 127.2, 128.5, 129.5, 142.5, 144.0, 144.4, 144.5, 167.8, 168.1, 169.6. ES/MS(+): *m/z*=941.541 (Calc. *m/z*=941.284) $[M+Na]^+$. HPLC (Method B in ESI): R_t=15.37 min.

N'-(pyridine-2-ylmethyl)-N^5-(4-sulfamoylphenylethyl)-2-(2-tritylthio)acetamido)pentanediamide, TrtMAGpy-ABS, H_2L^4Trt

Trt-MAGpy (**5**) was synthesized following the literature procedure[36]. Compound **5** (111 mg, 0.200 mmol) was added to DMF (5 mL) and mixed with ABS (40.0 mg, 0.200 mmol), EDC (37.8 mg, 0.200 mmol), HOBT (26.7 mg, 0.198 mmol) and TEA (6 µL). The reaction was left at RT for 1 day. The solvent was evaporated and the residue was washed with 0.1 M HCl (3×10 mL) and ethyl acetate (2×10 mL). The solid was dried under vacuum to give **H_2L^4Trt** (37.8 mg, 25.7%).

^{1}H NMR (d_6-DMSO, δ, 300 MHz): 1.77 (m, 2H), 1.96 (t, *J*=7.7Hz, 2H), 2.65 (t, *J*=6.8Hz, 2H), 2.76 (s, 2H), 3.14 (td, J_A=6.6Hz, J_B=19.3Hz, 2H), 4.09 (td, J_A=5.4Hz, J_B=7.8Hz, 1H), 4.24 (d, *J*=5.7Hz, 2H), 7.08-7.33 (m, 18H), 7.63 (d, *J*=8.0Hz, 2H) Ar-*H*; 7.86 (t, *J*=5.5Hz, 1H), 8.13 (d, *J*=7.6Hz, 1H), 8.36 (d, *J*=3.9Hz, 1H), 8.41 (t, *J*=5.7Hz, 1H). ^{13}C NMR (d_6-DMSO, δ, 75 MHz): 26.9, 31.5, 34.8, 35.7, 40.0, 41.8, 53.6, 66.9, 123.9, 124.3, 125.9, 126.7, 127.7, 129.1, 129.3, 141.6, 143.2, 143.8, 143.9, 144.1, 155.3, 170.2, 172.8, 173.2. ES/MS(+): *m/z*=736.2386 (Calc. *m/z*=736.2628) $[M+H]^+$. HPLC (Method A): R_t=12.04 min.

N-(2-oxo-2-((2-oxo-2-((2-oxo-2-((2-oxo-2-((5-sulfamoyl-1,3,4-thiadiazol-2yl)amino)ethyl)amino)ethyl)amino)ethyl)amino)ethyl)-2-(tritylthio)acetamide, TrtMAG$_3$-glyAZA, H_3L^5Trt

Trt-MAG$_3$ (**2**) (50.5 mg, 0.100 mmol) was added to DMF (5 mL) and mixed with **glyAZA** (35.1 mg, 0.100 mmol), EDC (20.0 mg, 0.105 mmol), HOBT (13.5 mg, 0.100 mmol) and TEA (20 µL). The reaction was left in ice bath for 1 h and then at RT for 1 day. The solvent was evaporated and the residue was re-dissolved in ethyl acetate (10 mL). The solution was washed with 1 M HCl, 3% $NaHCO_3$ and saturated brine, 20 mL each. The organic solution was collected and dried under vacuum. The raw product was recrystallized from ethanol/water to give **H_3L^5Trt** (41.0 mg, 56.6%).

^{1}H NMR (d_6-DMSO, δ, 300 MHz): 2.84 (s, 2H), 3.63 (d, *J*=5.6Hz, 2H), 3.71 (d, *J*=5.0Hz, 2H), 3.77 (d, *J*=5.9Hz, 2H), 4.08 (d, *J*=5.5Hz, 2H), 7.24-7.34 (m, 15H), 8.11-8.19 (m, 4H), 8.35 (s, 2H), 13.11 (s, 1H). ^{13}C NMR (d_6-DMSO, δ, 75 MHz): 36.3, 42.2, 42.4, 42.7, 66.4, 127.3, 128.5, 129.5, 144.5, 161.5, 164.9, 168.2, 169.4, 169.6, 170.0, 171.5. ES/MS(-): *m/z*=723.1169 (Calc. *m/z*=723.1478) $[M-H]^-$. HPLC (Method A): R_t=9.88 min.

N^5-(2-oxo-2-((5-sulfamoyl-1,3,4-thiadiazol-2-yl)amino)ethyl)-N^1-(pyridine-2-ylmethyl)-2-(2-(tritylthio)acetamido)pentanediamide, TrtMAGpy-glyAZA or H_2L^6trt.

Trt-MAGPy (5) (79.7 mg, 0.144 mmol) was mixed with BOP (76.4 mg, 0.173 mmol) and DIPEA (90 μL, 0.52 mmol) in DMF (5 mL) in ice bath for 20 min., then glyAZA (synthesised as above, (c) (60.7 mg, 0.173 mmol) was added. The mixture was stirred at RT overnight. The solvent was evaporated, and 3M HCl (20 mL) and ethyl acetate (20 mL) were used consecutively to wash off the impurities. The solid was further purified by silica gel chromatography using DCM/MeOH (9/1 by volume) to give H_2L^6Trt (32.3 mg, 29.0%).

^{1}H NMR (d_4-CD_3OD, δ, 500 MHz): 1.87-2.15 (m, 2H), 2.29-2.42 (m, 2H), 3.08 (d, J=1.26 Hz, 2H), 4.15-4.25 (m, 2H), 4.27 (dd, J_A=7.88, J_B=5.99 Hz, 1H), 4.53 (s, 2H), 4.61 (br, 2H), 7.09 - 7.52 (m, 17H), 7.80 (td, J_A=7.72, J_B=1.58 Hz, 1H), 8.48 (d, J=4.41 Hz, 1H). ^{13}C NMR (d_4-CD_3OD, δ, 125 MHz): 28.7, 32.6, 37.2, 43.6, 45.4, 54.7, 68.5, 123.0, 123.9, 128.2, 129.2, 130.8, 139.0, 145.6, 149.7, 158.9, 163.3, 166.6, 170.7, 171.4, 173.6, 175.7. ES/MS(+): m/z=795.1804 (Calc. m/z=795.1812) $[M+Na]^+$. HPLC (Method A): R_t=12.04 min.

Syntheses of rhenium complexes.

Sodium N-(2-oxo-2-((2-oxo-2-((4-sulfamoylphenylethyl)amido)ethyl)amido)ethyl)-2-thioacetamido-oxorhenium(V), Na[ReOMAG$_3$-ABS], Na[ReOL^2]

Trt-MAG$_3$ (**5**) (240 mg, 0.474 mmol) and sodium acetate (258 mg, 3.14 mmol) were mixed in dry methanol (60 mL), followed by addition of precursor $[ReOCl_3(PPh_3)_2]$ (395 mg, 0.474 mmol). After being refluxed for 4 h, the solution turned orange. After cooling, the solution was filtered and evaporated to dryness. The residue was chromatographed on silica gel with DCM/methanol. The orange eluent was collected and dried to give Na[ReO-MAG$_3$] **(6)** (185 mg, 80.6%). After **6** (48.4 mg, 0.100 mmol) was dissolved in phosphate buffer (10 mL, 0.1 M, pH=6.0), ABS (20.0 mg, 0.100 mmol) in acetonitrile (1 mL) was added to the solution, followed by EDC (19.1 mg, 0.100 mmol) and TEA (14 μL). The mixture was stirred at RT overnight. After the solvent was evaporated, the residue was chromatographed on silica gel with DCM/methanol (3/1 by volume). The orange eluent was collected and dried to give Na[ReOMAG$_3$-ABS] (37.1 mg, 55.6%).

^{1}H NMR (d_4-CD_3OD, δ, 500 MHz): 2.79-2.91(m, 2H), 3.41 (t, J=7.3Hz, 2H), 3.78 (d, J=17.2Hz, 1H), 4.02 (d, J=17.3Hz, 1H), 4.26 (d, J=18.0Hz, 1H), 4.28 (d, J=18.1Hz, 1H), 4.40 (d, J=18.0Hz, 1H), 4.65 (d, J=16.6Hz, 1H), 4.74 (d, J=18.2Hz, 1H), 4.99 (d, J=16.6Hz, 1H), 7.39 (d, J=8.3, 2H), 7.82 (d, J=8.2, 2H), ^{13}C NMR (d_4-CD_3OD, δ, 125 MHz): 34.3, 41.1, 41.5, 43.5, 49.9, 54.5, 57.7, 127.8, 130.5, 142.7, 144.0, 191.4, 194.7, 195.8. ES/MS(-): m/z=644.0281 (Calc. m/z=643.9832) $[M]^-$. IR: 978 cm^{-1} (Re=O), 1616 cm^{-1}(C=O). UV-Vis: λ_{abs}(MeOH)/nm (ε/ $M^{-1}\cdot cm^{-1}$)=224 (2.52×10^4), 274 (sh, 5.73×10^3), 339 (sh, 3.23×10^2), 402 (1.27×10^2), 485 (22). HPLC (Method C): R_t=3.03 min.

N,N'-(3-oxo-3-((4-sulfamoylphenylethyl)amido)propane-1,2-diyl)bis(2-thioacetamido)oxorhenium(V) Na[ReODADS-ABS], or Na[ReOL^3]

Na[ReODADS] (**7**) was synthesized from (**4**) according to the method described for Na[ReOMAG$_3$] (**6**); (50.1 mg, 70.5%). The title compound was synthesized by coupling **7** with ABS using the same method as described above for the MAG$_3$ system (35.8 mg, 51.5%). ^{1}H NMR (d_4-CD_3OD, δ, 500 MHz): 3.03 (m, 2H), 3.20 (t, J=7.4, 2H), 3.78 (d, J=17.4Hz, 1H), 3.84 (d, J=17.1 Hz, 1H), 4.14 (d, J=17.4Hz, 1H), 4.43 (d, J=16.9Hz, 1H), 4.75 (d, J=8.4Hz, 1H), 5.00 (d, J=12.9Hz, 1H), 7.50 (d, J=8.6, 2H), 7.91 (d, J=8.5, 2H), (*N.B.*: the ^{1}H resonance of the other NCH_2CHN proton was overlapped with other peaks). ^{13}C NMR (d_4-CD_3OD, δ, 125 MHz): 32.8, 40.1, 42.9, 43.5, 59.2, 69.6, 126.3, 129.1, 141.3, 142.4, 194.2, 195.8, 196.4. ES/MS(-): m/z=632.9900 (Calc. m/z=632.9941) $[M]^-$. IR: 962 cm^{-1}(Re=O), 1581 cm^{-1}(C=O). UV-Vis: λ_{abs}(MeOH)/nm (ε/ $M^{-1}\cdot cm^{-1}$)=226 (2.93×10^4), 249 (sh, 1.10×10^4), 300 (sh, 3.15×10^3), 332 (sh, 1.58×10^3), 402 (1.83×10^2), 489 (11). HPLC (Method 3): R_t=4.27 min.

N-(2-oxo-2-((2-oxo-2-((2-oxo-2-((2-oxo-2-((5-sulfamoyl-1,3,4-thiadiazol-2yl)amido)ethyl)amido)ethyl)amido)ethyl)amido)ethyl)-2-(thio)acetamido Na[ReOMAG$_3$-glyAZA] or **Na[ReOL^5]**

The title compound was synthesized by coupling **6** with glyAZA using an analogous method to that described for the synthesis of (45.1 mg, 64.1%). ^{1}H NMR (d_4-CD_3OD, δ, 500 MHz): 2.91 (d, J=6.9, 2H), 3.82 (d, J=17.2Hz, 1H), 4.05 (d, J=17.2Hz, 1H), 4.29 (d, J=18.6 Hz, 1H), 4.32 (d, J=18.8Hz, 1H), 4.42 (d, J=18.2Hz, 1H), 4.73 (d, J=17.1Hz, 1H), 4.79 (d, J=18.1Hz, 1H), 5.05 (d, J=17.1Hz, 1H). ^{13}C NMR (d_4-CD_3OD, δ, 125 MHz): δ(ppm)=41.1, 43.6, 54.5, 56.1, 57.7, 164.61, 191.5, 194.7, 195.8. ES/MS(-): m/z=680.9280 (Calc. m/z=680.9650) $[M]^-$. IR: 977 cm^{-1}(Re=O), 1629 cm^{-1}(C=O). UV-Vis: λ_{abs}(MeOH)/nm (ε/ $M^{-1}\cdot cm^{-1}$)=221 (1.43×10^4), 270 (sh, 4.61×10^3), 337 (sh, 3.18×10^2), 397 (1.03×10^2), 491 (18). HPLC (Method A): R_t=6.62 min.

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5-[4-(*N*-propyl benzamide)]-10,15,20-tri(4-pyridyl)-porphyrin, [TPyP-propyl, H_2L^7]

4-(10,15,20-Triphenylporphyrin-5-yl)-benzoic acid[37] and 5-(4-Carboxyphenyl)-10,15,20-tris(4-pyridyl)porphyrin [38], were synthesised according to literature methods with analytical data in accord with the literature values (ESI).

5-[4-(*N*-propyl benzamide)]-10,15,20-tri(4-pyridyl)-porphyrin was synthesised employing general amide coupling method B (ESI). 5-(4-Carboxyphenyl)-10,15,20-tris(4-pyridyl)porphyrin 9 (75 mg, 0.113 mmol), DIPEA (38.7 mg, 52.3 µL 0.300 mmol), propylamine (7.97 mg, 8.04 µL, 0.135 mmol), BOP (67.5 mg, 0.146 mmol), $CHCl_3$ (10 mL). 5-[4-(*N*-propyl benzamide)]-10,15,20-tri(4-pyridyl)-porphyrin was purified *via* silica gel chromatography (0 % to 5 % MeOH in $CHCl_3$). Yield: 65.2 mg, 0.093 mmol, 82 %.

^{1}H NMR (300 MHz, d_6–DMSO, 25 ºC): δ 9.40 (d, 6H, *J* = 6.1, C*H*$_{py}$), 9.14 (m, 8H, C*H*$_{pyrr}$), 8.91 (m, 6H, C*H*$_{py}$), 8.65 (t, 1H, *J* = 5.5, CON*H*), 8.27 (d, 2H, *J* = 7.9, Ar*H*), 8.15 (d, 2H, *J* = 8.1, Ar*H*), 3.52 (m, 2H, CONHC*H*$_2$CH$_2$CH$_3$), 1.81 (m, 2H, CONHCH$_2$C*H*$_2$CH$_3$), 1.07 (t, 2H, *J* = 6.9, CONHCH$_2$CH$_2$C*H*$_3$), -2.95 (br s, 2H, ring N*H*). ESI-MS calcd for $C_{44}H_{35}N_8O$ [M + H]$^+$ 703.2928, found 703.2935

5-[4-(*N*-propyl benzamide)]-10,15,20-tri(4-pyridyl)-porphyrinchloroindium(III), $InClL^7$

This was prepared using general metal complexation method 1. H_2L^7 (50.0 mg, 0.072 mmol, NaOAc (35.0 mg, 0.429 mmol, $InCl_3$ (32.0 mg, 0.143 mmol), HOAc (10 ml). Yield: 51.2 mg, 0.061 mmol, 84%

1H NMR: (300 MHz, d_6-DMSO, 25^{o}C): 8.99 (s, 8H, CH$_{pyr}$), 8.50 (t, 1H, *J* = 5.4, CONH), 8.31 (2xd, 2x2H, ArH), 8.22 (m, 6H, o-Ph), 7.85 (m, 9H, o,m,p-Ph), 3.43 (m, 2H, CONH*CH$_2$*CH$_2$CH$_3$), 1.75 (m, 2H, CONHCH$_2$*CH$_2$*CH$_3$*)*, 1.02 (t, 3H, CONHCH$_2$CH$_2$*CH$_3$*). ESI-MS Calculated for $C_{48}H_{36}InN_5O$ [M – Cl]$^+$, 812.1875, found 812.1855; HPLC: (method A in ESI): R_t = 10.64 min.

5-[4-(N-(4-Sulphamoylphenethyl)benzamide)]-10,15,20-triphenylporphyrin, [H_2TPP-ABS], H_2L^8

The title compound was synthesised employing general peptide coupling method 1. **8** (250 mg, 0.331 mmol), 4-(2-aminoethyl)benzenesulfonamide (100 mg, 0.500 mmol), DIPEA (64.6 mg, 87.1 µL, 0.500 mmol), DMF (10 mL). Purification was achieved by silica gel chromatography (5 % MeOH in $CHCl_3$). Yield: 236 mg, 0.281 mmol, 85 %.

^{1}H NMR (300 MHz, d_6–DMSO, 25 ºC): δ 8.99 (t, 1H, *J* = 5.5, CON*H*), 8.81 (m, 8H, C*Hpyrr*), 8.29 (d, 2H, *J* = 8.1, Ar*H*), 8.23 (d, 2H, *J* = 8.4, Ar*H*), 8.17 (m, 6H, *o*-Ar*H*), 7.84 (d, 2H, *J* = 8.4, Ar*H*), 7.77 (m, 9H, *m,p*-Ar*H*), 7.55 (d, 2H, *J* = 8.4 Ar*H*), 7.37 (s, 2H, SO$_2$N*H*$_2$), 3.69 (m, 2H, CONHC*H*$_2$CH$_2$), 3.06 (t, 1H, *J* = 7.2, CONHCH$_2$C*H*$_2$). ^{13}C NMR (125 MHz, d_6–DMSO, 25 ºC): δ 166.3, 144.0, 143.9, 142.2, 141.2, 134.2, 134.1, 131.0 (bs), 129.3, 128.1, 127.0, 125.9, 120.3, 120.2, 119.1, 34.9, 25.3. Mass Spectrum ESI-MS calcd for $C_{53}H_{40}N_6O_3S$ [M + H]$^+$ 841.2961, found 841.2768. HPLC: (Method A) R_t = 11.81 min. Elem. Anal.: Found: C; 75.2 %, H; 4.9 %, N; 10.1 %. Calc.: C; 75.7 %, H; 4.8 %, N; 10.0 %.

5-[4-(N-(2-Oxo-2-(5-sulphamoyl-1,3,4-thiadiazol-2-ylamino)ethyl)benzamide)]-10,15,20-triphenyl-porphyrin, [H_2TPP-AZA], H_2L^{11}

The title compound was synthesised employing general amide coupling method 1. **8** (330 mg, 0.436 mmol), **glyAZA** (180 mg, 0.512 mmol), DIPEA (66.2 mg, 89.2 µL, 0.512 mmol), DMF (10 mL). Purification was achieved via silica gel chromatography (2 to 8 % MeOH in $CHCl_3$). Yield: 256 mg, 0.292 mmol, 67 %.

^{1}H NMR (500 MHz, d_6–DMSO, 25 ºC): δ 13.39 (s, 1H, CON*H*-thiadiazole), 9.44 (t, 1H, *J* = 5.5, CON*H*), 8.84 (m, 8H, C*H*$_{pyrr}$), 8.40 (s, 2H, SO$_2$N*H*$_2$), 8.37 (m, 4H, Ar*H*), 8.23 (m, 6H, *o*-Ar*H*), 7.84 (m, 9H, *m,p*-Ar*H*), 4.45 (d, 2H, *J* = 5.6, CONHC*H*$_2$CONH), -2.90 (s, 2H, ring N*H*). ^{13}C NMR (125 MHz, d_6–DMSO, 25 ºC): δ 169.6, 166.8, 164.5, 161.3, 144.5, 141.1, 134.2, 134.2, 133.0, 131.5, 128.1, 127.0, 126.1, 120.3, 120.1, 118.9, 43.0. ESI-MS calcd for $C_{49}H_{36}N_9O_4S_2$ [M + H]$^+$ 878.2326, found 878.2275. HPLC: (Method A) R_t = 10.82 min. Elem. Anal.: Found: C; 66.8 %, H; 3.8 %, N; 14.4 %. Calc.: C; 67.0 %, H; 4.0 %, N; 14.4 %.

5-[4-(N-(4-Sulphamoylphenethyl)benzamide)]-10,15,20-triphenylporphyrin(chloroindium(III)), [In(Cl)TPP-ABS] or [$InClL^8$]

The title compound was synthesised employing general metal complexation method 1. **H_2L^8** (74.5 mg, 0.087 mmol), NaOAc (43.6 mg, 0.532 mmol), $InCl_3$ (39.2 mg, 0.177 mmol), AcOH (10 mL). A maroon coloured solid was obtained. Yield: 69.7 mg, 0.070 mmol, 81 %.

^{1}H NMR (300 MHz, d_6–DMSO, 25 ºC): δ 9.11 (t, 1H, *J* = 5.6, CON*H*), 9.13-9.01 (m, 8H, C*H$_{pyrr}$*), 8.26 (d, 2H, *J* = 7.8, Ar*H*), 8.19 (m, 8H, *o*-Ph, Ar*H*), 7.95 (d, 2H, *J* = 8.2, Ar*H*), 7.89 (m, 9H, *m,p*-Ph), 7.51 (d, 2H, *J* = 8.0, Ar*H*), 3.71 (m, 2H, CONHC*H*$_2$CH$_2$), 3.04 (t, 2H, *J* = 7.6, CONHCH$_2$C*H*$_2$). ^{13}C NMR (75.5 MHz, d_6–DMSO, 25 ºC): δ 166.1, 148.0, 147.8, 147.7, 147.4, 144.5 144.1, 141.9, 141.3, 134.4, 134.2, 133.9, 132.54, 132.51, 132.4, 130.5, 128.1, 127.1, 125.9, 125.8, 121.5, 121.3, 120.1, 38.2. 27.4. ESI-MS calcd for $C_{53}H_{38}InN_6O_3S$ [M - Cl]$^+$ 953.1765, found 953.1789. HPLC: (Method A) R_t = 10.12 min. Elem. Anal.: Found: C; 63.9 %, H; 4.0 %, N; 8.7 %. Calc.: C; 64.4 %, H; 3.9 %, N; 8.5 %.

5-[4-(N-(4-Sulphamoylphenethyl)benzamide)]-10,15,20-triphenylporphyrin(chlorogallium(III)), [Ga(Cl)TPP-ABS] or [GaClL^8]

The title compound was synthesised employing general metal complexation method 1. **H$_2$L^8** (68.5 mg, 0.082 mmol), NaOAc (40.1 mg, 0.489 mmol), $GaCl_3$ (28.7 mg, 0.163 mmol), AcOH (10 mL). A maroon coloured solid was obtained. Yield: 57.3 mg, 0.061 mmol, 74 %.

^{1}H NMR (500 MHz, d_6–DMSO, 25 ºC): δ 8.92 (m, 8H, C*H$_{pyrr}$*), 8.76 (t, 1H, *J* = 5.5, CON*H*), 8.32 (d, 2H, *J* = 7.6, Ar*H*), 8.21 (d, 2H, *J* = 7.7, Ar*H*), 8.26 (m, 6H, *o*-Ph), 7.88 (d, 2H, *J* = 8.1, Ar*H*), 7.85 (m, 9H, *m,p*-Ph), 7.49 (d, 2H, *J* = 7.9, Ar*H*), 3.75 (m, 2H, CONHC*H*$_2$CH$_2$), 3.15 (t, 2H, *J* = 7.3, CONHCH$_2$C*H*$_2$). ESI-MS calcd for $C_{53}H_{38}GaN_6O_3S$ [M - Cl]$^+$ 907.1982, found 907.1965. HPLC: (Method A) R_t = 10.05 min. Elem. Anal.: Found: C; 67.8 %, H; 4.3 %, N; 8.7 %. Calc.: C; 67.5 %, H; 4.1 %, N; 8.9 %.

5-[4-(N-(2-Oxo-2-(5-sulphamoyl-1,3,4-thiadiazol-2-ylamino)ethyl)benzamide)]-10,15,20-triphenyl-porphyrin(chloroindium(III)), [In(Cl)TPP-AZA] or [InClL^{11}]

The title compound was synthesised employing general metal complexation method 1 (ESI). **H$_2$L^{11}** (40.0 mg, 0.046 mmol), NaOAc (22.0 mg, 0.273 mmol), $InCl_3$ (19.7 mg, 0.091 mmol), AcOH (10 mL). A purple coloured solid was obtained. Yield: 34.0 mg, 0.033 mmol, 72 %.

^{1}H NMR (500 MHz, d_6–DMSO, 25 ºC): δ 9.06 - 9.03 (m, 2H, C*H$_{pyrr}$*), 8.99 (m, 7H, C*H$_{pyrr}$*, CON*H*), 8.43 (d, 2H, *J* = 8.1, Ar*H*), 8.36 (d, 2H, *J* = 8.3, Ar*H*), 8.25 (m, 6H, *o*-Ar*H*), 7.90 (m, 9H, *m-,p*-Ar*H*), 4.20 (d, 2H, *J* = 5.9, CONHC*H*$_2$). ^{13}C NMR (125 MHz, d_6–DMSO, 25 ºC): δ 175.8, 175.0, 172.1, 165.9, 148.12, 148.09, 148.06, 147.8, 144.1, 141.4, 134.5, 134.4, 134.2, 132.6, 132.5, 132.5, 128.2, 127.0, 125.9, 121.4, 121.3, 120.3, 45.5. Mass Spectrum ESI-MS calcd for $C_{49}H_{33}InN_9O_4S_2$ [M - Cl]$^+$ 990.1130, found 990.1090. HPLC: (Method A) R_t = 9.99 min.

5-[4-(N-(2-Oxo-2-(5-sulphamoyl-1,3,4-thiadiazol-2-ylamino)ethyl)benzamide)]-10,15,20-triphenyl-porphyrin(chlorogallium(III)), [Ga(Cl)TPP-AZA] or [GaClL^{11}]

The title compound was synthesised employing general metal complexation method 1. **H$_2$L^{11}** (50.0 mg, 0.057 mmol), NaOAc (28.0 mg, 0.341 mmol), $GaCl_3$ (21.0 mg, 0.114 mmol), AcOH (10 mL). A maroon coloured solid was obtained. Yield: 45.3 mg, 0.046 mmol, 81 %.

^{1}H NMR (500 MHz, d_6–DMSO, 25 ºC): δ 9.13-8.91 (m, 8H, C*H$_{pyrr}$*), 8.76 (br s, 1H, CON*H*), 8.40 (d, 2H, *J* = 7.3, Ar*H*), 8.31 (d, 2H, *J* = 7.5, Ar*H*), 8.22 (m, 6H, *o*-Ar*H*), 7.86 (m, 9H, *m-p*-Ar*H*), 4.20 (d, 2H, *J* = 4.9, CONHC*H*$_2$). ^{13}C NMR (75.5 MHz, d_6–DMSO, 25 ºC): δ 165.9, 147.3, 147.0, 143.6, 140.8, 134.2, 132.3, 132.2, 128.4, 127.2, 126.1, 120.4, 119.4, 45.1. ESI-MS calcd for $C_{49}H_{33}GaN_9O_4S_2$ [M - Cl]$^+$ 944.1347, found 944.1334. HPLC: (Method A) R_t = 10.11 min. Elem. Anal.: Found: C; 60.5 %, H; 3.6 %, N; 12.2 %. Calc.: C; 60.0 %, H; 3.4 %, N; 12.8 %.

5-[4-(N-(4-Sulphamoylphenethyl)benzamide)]-10,15,20-tri(4-sulfonylphenyl)porphyrin, [H$_2$TSPP-ABS], H$_2$L^9

The title compound was synthesised employing the general sulfonation method (ESI). **H$_2$L^7** (70.0 mg, 0.083 mmol), conc. H_2SO_4 (2 mL). Yield : 66.6 mg, 0.058 mmol, 70 %.

^{1}H NMR (500 MHz, d_6–DMSO, 25 ºC): δ 9.05 (t, 1H, *J* = 5.7, CON*H*), 8.82 (m, 8H, C*H*pyrr), 8.29 (d, 2H, *J* = 8.0, Ar*H*), 8.21 (m, 6H, Ar*H$_{SO3Na}$*), 8.17 (d, 2H, *J* = 8.1 Hz, Ar*H*), 8.05 (m, 6H, Ar*H$_{SO3Na}$*), 7.87 (d, 2H, *J* = 8.1, Ar*H*), 7.61 (d, 2H, *J* = 7.8, Ar*H*), 7.37 (s, 2H, SO$_2$N*H*$_2$), 3.71 (m, 2H, CONHC*H*$_2$CH$_2$), 3.14 (t, 1H, *J* = 7.5, CONHCH$_2$C*H*$_2$). ^{13}C NMR (75.5 MHz, d_6–DMSO, 25 ºC): δ 166.1, 147.8, 144.1, 143.8, 142.5, 141.1, 134.24, 134.16, 133.7, 131.4 (broad), 131.2, 129.1, 125.9, 124.6, 120.1, 119.8, 118.9, 35.6, 26.4. ESI-MS calcd for $C_{53}H_{37}N_6O_{12}S_4$ [M – 3Na]$^{3-}$ /3 359.0451, found 359.0467. HPLC: (Method B, ESI) R_t = 15.20 min.

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5-[4-(N-(2-Oxo-2-(5-sulphamoyl-1,3,4-thiadiazol-2-ylamino)ethyl)benzamide)]-10,15,20-tri(4-sulfonylphenyl)-porphyrin, [H_2TSPP-AZA] or H_2L^{12}

The title compound was synthesised employing the general sulfonation method (ESI). H_2L^{10} (50.0 mg, 0.057 mmol), conc. H_2SO_4 (2 mL). Yield : 48.6 mg, 0.041 mmol, 72 %.

^{1}H NMR (500 MHz, d_6–DMSO, 25ºC): δ 8.90 (t, 1H, *J* = 5.7, CON*H*), 8.88 (m, 8H, CH_{pyrr}), 8.37 (m, 4H, Ar*H*), 8.21 (d, 6H, *J* = 8.1, ArH_{SO3Na}), 8.07 (d, 6H, *J* = 8.0, ArH_{SO3Na}), 4.20 (d, 2H, *J* = 5.6, CONHCH_2CONH), -2.91 (s, 2H, ring N*H*). ^{13}C NMR (125 MHz, d_6–DMSO, 25 ºC): 174.9, 171.9, 165.9, 165.3, 147.8, 143.9, 141.2, 134.3, 134.2, 133.7, 131.6 (broad), 125.9, 124.2, 119.8, 119.7, 119.3, 45.5. ESI-MS calcd for $C_{49}H_{32}N_9O_{13}S_5$ $[M – 3Na]^{3-}/3$ 371.3580, found 371.3576. HPLC: (Method B, ESI) R_t = 16.11 min.

5-[4-(*N*-(4-Sulphamoylphenethyl)benzamide)]-10,15,20-tri(4-*N*-methylpyridinium phenyl)-porphyrin tri-iodide, [H_2TMePyP-ABS], H_2L^{10}

H_2L^{10} was synthesised employing the general alkylation method (ESI). The tripyridylporphrin ABS conjugate, synthesis described above (65.0 mg, 0.077 mmol), MeI (109 mg, 47.9 µL, 0.77 mmol), DMF. Yield: 114 mg, 0.090 mmol, 92 %.

^{1}H NMR (500 MHz, d_6–DMSO, 25ºC): 9.46 (m, 6H, CH_{py}), 9.11 (m, 8H, CH_{pyrr}), 9.02 (t, 1H, *J* = 5.4, CON*H*), 8.99 (m, 6H, CH_{py}), 8.32 (d, 2H, *J* = 8.7, Ar*H*), 8.30 (d, 2H, *J* = 8.8, Ar*H*), 7.81 (d, 2H, *J* = 8.3, Ar*H*), 7.56 (d, 2H, *J* = 8.4, Ar*H*), 7.34 (s, 2H, SO_2NH_2), 4.70 (m, 3x3H, 3xN-CH_3), 3.70 (m, 2H, CONHCH_2CH$_2$), 3.07 (t, 2H, *J* = 6.9, CONHCH$_2$CH_2), -3.06 (m, 1H) ^{13}C NMR (125 MHz, d_6–DMSO, 25ºC): δ 166.2, 156.6, 156.5, 144.3, 144.2, 143.9, 143.1, 142.1, 134.4, 134.3, 132.1, 129.3, 126.0, 125.8, 124.1, 121.9, 115.4, 114.7, 114.5, 111.8, 47.9, 34.5, 25.3. ESI-MS calcd for $C_{53}H_{44}N_9O_3S$ $[M – 3I]^{3+}/3$ 269.1143, found 269.1143, ESI-MS calcd for $C_{53}H_{44}N_9O_3S$ $[M – 3I]^{3+}/2$ 444.1717, found 444.1708. HPLC: (Method C) R_t = 12.22 min. Elem. Anal.: Found: C; 50.3 %, H; 3.3 %, N; 9.8 %. Calc. [H2L10 + MeOH]: C; 49.9 %, H; 3.7 %, N; 9.7 %.

5-[4-(N-(2-Oxo-2-(5-sulphamoyl-1,3,4-thiadiazol-2-ylamino)ethyl)benzamide)]-10,15,20-tri(4-N-methylpyridiumyl)-porphyrin tri-iodide, [H_2TMePyP-AZA]I_3 or H_2L^{13}

Compound H_2L^{13} was synthesised employing the general alkylation method described in ESI. [H_2TPyP-AZA] (50.0 mg, 0.038 mmol), MeI (54.3 mg, 23.8 µL, 0.38 mmol), DMF. Yield: 52.9 mg, 0.040 mmol, 88 %.

^{1}H NMR (300 MHz, d_6–DMSO, 25 ºC): δ 9.46 (d, 6H, *J* = 6.6, CH_{py}), 9.36 (t, 1H, *J* = 5.4, CON*H*), 9.26 - 9.05 (m, 8H, CH_{pyrr}), 8.93 - 9.03 (m, 6H, CH_{py}), 8.40 (d, 2H, *J* = 8.0, Ar*H*), 8.36 (d, 2H, *J* = 7.8, Ar*H*), 4.71 (2s, 3x3H, 3xN-CH_3), 4.43 (d, 2H, *J* = 5.8, CONHCH_2CONH), -3.05 (br s, 1H, ring N*H*). Mass Spectrum ESI-MS calcd for $C_{49}H_{41}N_{12}O_4S_2$ $[M – 3I]^{3+}/3$ 308.0640, found 308.0621, ESI-MS calcd for $C_{49}H_{41}N_{12}O_4S_2$ $[M – 3I]^{3+}/2$ 462.0960, found 462.0993. HPLC: (Method C) R_t = 13.08 min.

5-[4-(*N*-(4-Sulphamoylphenethyl)benzamide)]-10,15,20-tri(4-sulfonylphenyl)-porphyrin(chloroindium (III)), [In(Cl)TSPP-ABS], [InClL^9]

The title compound was synthesised employing general metal complexation method 3. H_2L^9 (40.0 mg, 0.035 mmol), $InCl_3$ (15.5 mg, 0.070 mmol) in pH 4.5 NaOAc buffer (5 mL). Yield: 29.9 mg, 0.023 mmol, 66 %.

^{1}H NMR (300 MHz, d_6–DMSO, 25 ºC): δ 9.07 (t, 1H, *J* = 5.4, CON*H*), 8.94 (s, 8H, CH_{pyrr}), 8.29 (s, 4H, Ar*H*), 8.18 (d, 6H, *J* = 8.0, ArH_{SO3Na}), 8.08 (d, 6H, *J* = 8.2, ArH_{SO3Na}), 7.60 (d, 2H, *J* = 8.0, Ar*H*), 7.32 (d, 2H, *J* = 8.0, Ar*H*), 3.65 (m, 2H,CONHCH_2CH$_2$), 3.00 (t, 2H, *J* = 7.4, CONHCH$_2$CH_2) ^{13}C NMR (75.5 MHz, d_6–DMSO, 25 ºC): δ 166.3, 147.72, 147.69, 147.5, 147.4, 145.9, 144.1, 141.7, 140.3, 134.2, 134.0, 132.6 (broad), 128.1, 125.9, 125.7, 124.2, 120.8, 120.4, 35.0, 25.0. Mass Spectrum ESI-MS calcd for $C_{53}H_{35}ClInN_6O_{12}S_4$ $[M – 3Na]^{3-}/3$ 408.3313, found 408.3345. HPLC: (Method B) R_t = 13.23 min.

5-[4-(*N*-(4-Sulphamoylphenethyl)benzamide)]-10,15,20-tri(4-*N*-methylpyridiniumylphenyl)-porphyrin (chloroindium(III)) trichloride [In(Cl)TMePyP-ABS]Cl_3, [InClL^{10}]

The title compound was synthesised employing general metal complexation method 3. H_2L^{10} (50.0 mg, 0.039 mmol), $InCl_3$ (17.4 mg, 0.079 mmol) in pH 4.5 NaOAc buffer (5 mL). Yield: 30.3 mg, 0.027 mmol, 68 %.

^{1}H NMR (500 MHz, d_6–DMSO, 25 ºC): δ 9.41 - 9.67 (m, 6H, CH_{py}), 9.23 - 9.35 (br s, 4H CH_{pyrr}), 9.13 - 9.23 (br s, 3H, CON*H*, CH_{pyrr}), 9.02 - 9.11 (br s, 2H, CH_{pyrr}), 8.87 - 9.00 (br s, 6H, CH_{py}), 8.36 (d, 2H, *J* = 7.5, Ar*H*), 8.30 (d, 2H, *J* = 7.5, Ar*H*), 7.81 (d, 2H, *J* = 8.0, Ar*H*), 7.55 (d, 2H, *J* = 8.0, Ar*H*), 7.36 (s, 2H, SO_2NH_2), 4.66 - 4.87 (br s, 9H, 3xCH_3), 3.69 (m, 2H, CONHCH_2CH$_2$), 3.09 - 3.16 (t, 2H, *J* = 5.7, CONHCH$_2$C*H*$_2$). ^{13}C NMR (75.5 MHz, *d*$_6$–DMSO, 25 ºC): δ 166.7, 156.6, 156.5, 147.8, 146.5, 146.4, 144.8, 144.3, 144.2, 142.9, 133.8, 133.3, 132.4, 132.3, 129.8, 126.2, 126.1, 117.3, 116.8, 48.1, 47.9, 35.1, 26.2. Mass Spectrum ESI-MS calcd for $C_{53}H_{42}ClInN_9O_3S$ [M – 3Cl]$^{3+}$/3 344.7281, found 344.7298. HPLC: (Method C in ESI) R_t = 12.05 min.

General method for copper-64 radiolabelling

^{64}Cu-ATSM was prepared from H_2-ATSM according to a published method[39].[2] ^{64}Cu**L** was prepared by adding $^{64}Cu(OAc)_2$(aq) (70 µL of a 1 MBq/µL solution) to a solution of H_2**L** in DMSO (100 µL, 1 mg/mL). After 20 min stirring at room temperature HPLC analysis was carried out using a Platinum C18 column (5 µm particle size, dimensions 250 x 3.0 mm). A 20 min gradient method with acetonitrile/water as the mobile phase was used: 0 min 5% MeCN; 10 min 95% MeCN; 15 min 95% MeCN; 18 min 5% MeCN, 20 min 5% MeCN. Flow rate = 1.0 mL/min. The radiochemical purity of ^{64}Cu**L** (R_t = mins) was 99%. 0.9% saline solution (to < 5% v/v ethanol) for use in PET imaging experiments. The specific activity of the administered tracer was approximately 20 MBq/mL (0.2 MBq/µg of H_2L).

General method for indium-111 radiolabelling

The ligand was prepared as a 1.0 mg/mL solution in DMSO or distilled water. For indium labeling of water soluble porphyrins 10 µL of the stock porphyrin solution was diluted with 90 µL of pH 4.5 sodium acetate buffered solution and heated at 115 °C for 30 min in an Eppendorf tube in the presence of 20 µL ^{111}InCl3 (< 10 MBq per experiment). For indium labelling of tetraphenyl porphyrin derivative 10 µL of the stock solution was diluted with 90 µL of AcOH and heated at 120 °C for 1 h in the presence 10 µL of a 0.1 M NaOAc (aq.) solution and 20 µL $^{111}InCl_3$ (< 10 MBq per experiment). AcOH was then driven off under nitrogen and the residue re-suspended in 50 µL of $CHCl_3$. The $CHCl_3$ layer was washed repeatedly with 100 µL aliquots of distilled water until no further activity was associated with the aqueous washings. For all porphyrin-based compounds the 20 min HPLC gradient methods (analytical HPLC) of was performed by one of three methods below:

General method for technetium-99m radiolabelling

The radiolabelling was carried out according to the procedure described previously[40], using $[^{99m}TcO_4]^-$ with $SnCl_2$ as the reducing agent in sodium carbonate buffer at a temperature of 95 °C. Briefly, the ligand (L^2 to L^6, 0.1 mg) was dissolved in TFA (190 µL) and treated with triethylsilane (10 µL) to remove the Trt-group. After removal of the solvent under a stream of N_2, sodium carbonate solution (80 µL, 0.1 mol/L, pH=9.5) was added to the residue. Next, $SnCl_2$ solution (3 µL, 1 mg/mL) in citrate buffer (0.1 mol/L, pH=5.0) and $[^{99m}TcO_4]^-$ (200 µL, 20 MBq) solution were added, and the reaction mixture was vigorously stirred and allowed to react at 95 °C for 1 h. The radiochemical purity and labelling efficiency were determined using radio-HPLC (Method C in ESI) or radio-TLC. For radio-TLC, acetonitrile/water (1/1 by volume) was used for the determination of ^{99m}Tc-colloid in the silica gel TLC (R_f=0) and 2-butanone/ethyl acetate (2/3 by volume) was used for the determination of ^{99m}Tc-pertechnetate (R_f=1.0) [4]. In the cases when the radiochemical purity of the radiotracer did not exceed 95%, the raw product was purified using a C18 Sep-Pak™ cartridge. The cartridge was pre-treated using ethanol (5 mL), followed by water (5 mL), before the raw product was loaded. The loaded cartridge was rinsed twice with water (2×5 mL) to release the ^{99m}Tc-pertechnetate and any other hydrophilic impurities, and the technetium-labelled product was eluted with ethanol (1 mL). The radiochemical purity of the purified radiotracer was then determined using the method described above. All the ^{99m}Tc complexes had RCP's of >98% after purification.

Confocal fluorescence microscopy

HCT116 cells were seeded as monolayers in T75 tissue culture flasks, and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % foetal bovine serum, L-glutamine, penicillin and streptomycin. Cells were maintained at 37 °C in a 5 % CO2 humidified atmosphere and grown to approximately 85 % confluence before being split using 2.5 % trypsin. For microscopy, cells were seeded onto chambered cover-glass slides and incubated for 12 h to ensure adhesion.
Porphyrin complexes were prepared as 10 mM solution in DMSO or distilled water, and diluted to 10 µM with DMEM, and incubated with the cells at 37 °C. Prior to imaging, the solution was replaced with 1 mL fresh DMEM. Background autofluorescence was measured by imaging the cells in 1 mL of DMEM medium only. The fluorescent uptake of Porphyrin complexes was imaged by laser-scanning confocal microscopy using a Zeiss LSM 510 META microscope irradiating at 405nm with emission filtered between 565 and 615 nm.

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Notes and references

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