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THE ENZYME-INHIBITOR APPROACH TO CELL-SELECTIVE LABELLING. III. SULPHONAMIDE INHIBITORS OF CARBONIC ANHYDRASE AS CARRIERS FOR RED CELL LABELLING

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Selective radiolabelling of red blood cells via an enzyme-inhibitor approach represents a novel method in diagnostic nuclear medicine. Current problems in blood pool labelling could be overcome by using selective sulphonamide inhibitors as carriers. Red cell carbonic anhydrase is identified as an ideal target enzyme for such an approach. A brief review of the target enzyme is presented together with the screening of a series of synthesised sulphonamide inhibitors. p-Iodobenzenesulphonamide, 4-[(4-iodophenyl)thio]benzenesulphonamide and 5-(4-bromophenyl)sulphonyl]thiophene-2-sulphonamide were found to be particularly potent, reversible, lipophilic inhibitors of carbonic anhydrase, characteristics that warrant their further investigation as potential carriers. 4-Iodo-3-(iodoacetamido)benzenesulphonamide was a moderate inhibitor but caused relatively fast irreversible inactivation, making it a candidate for longer term studies.

KEY WORDS: Carbonic Anhydrase, Enzyme-inhibitor approach, red cell labelling.

INTRODUCTION

Diagnostic nuclear medicine involves the non-invasive imaging of the distribution of an administered radionuclide. The most useful information is obtained when the radioisotope is localised to the organ or tissues of interest. A rational to achieve this selective radiolabelling of cells must involve the use of carrier ligands which bind to cell-specific acceptors. Appropriate ligands belonging to Eckelman's 'substrate specific class' include enzyme inhibitors, antibodies, metabolites, hormones, neurotransmitters and their chemical analogues.[1] While examples of all these types of ligand have been reported,[2] we have been particularly interested in the application of the first class, the enzyme inhibitors, to the selective labelling of red blood cells (rbc). A review of this general approach is presented below, together with the potential benefits of its application to blood pool imaging. In addition, the characteristics of the relevant enzyme-inhibitor interactions are discussed to emphasise that a logical choice of radiolabel carrier can be made.

Enzyme Inhibitors as Carriers

This approach has been admirably reviewed.[3,4,5] It appears to have been first used in humans in the late 1960's,[6] but has found rather limited application. This is because

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the constraints which determine its feasibility are particularly limiting:

1. There must be a relatively high concentration of enzyme in the target tissue. It may be necessary to conserve a high degree of enzyme activity while still targeting 50 kBq of radionuclide per cm^3 of tissue, for scintigraphic visualisation. The absolute enzyme concentration will then determine the requisite specific activity of the labelled inhibitor.
2. There must be a significantly higher concentration of the enzyme in the target tissue, unless kinetic accessibility dominates. In this last instance, for example, an inhibitor administered i.v. may label blood cells rapidly before having a chance to diffuse to tissues where the target enzyme predominates.
3. The radiolabelled inhibitor must bind tightly to the enzyme. The ratio of the enzyme concentration to the enzyme-inhibitor dissociation constant ($[E_o]/K_D$) effectively determines the suitability of the system, since in the limit, this equals the localisation index, i.e. the bound to free ratio. A lower enzyme concentration thereby necessitates a higher affinity for the inhibitor. Tight binding may still be required when the enzyme concentration is not low to prevent fast dissociation and washout.
4. The enzyme must be accessible to the inhibitor.
5. The enzyme should be purifiable from the target tissue/cells for preliminary *in vitro* screening of suitable inhibitors.

The few principal examples which have been reported are listed in Table I. However, none are ideal. For example, acetylcholinesterase is present at a low concentra-

TABLE I
Some enzyme inhibitors used in cell-selective labelling

Target Enzyme	Inhibitor	K_D(mol dm^{-3})	Application
Dihydrofolate reductase	[^{131}I]-3′-Iodoaminopterin[6]	$< 6 \times 10^{-10}$	Tumor imaging
11β-Hydroxlase	[^{3}H]-Metyrapone[11]	–	
	[^{3}H]-Metyrapol[5,8]	8×10^{-9}	Adrenal cortex localisation
	[^{14}C]-Isoxazole*[12]	–	
	[^{3}H]-3-Iodo-SKF-12185*[11]	–	
	[^{125}I]-19-Iodocholesterol*[13]	–	
	[^{125}I]-3-Iodo-SKF-12185[11]	–	
	[^{123}]-3-Iodo-SKF-12185[11,14]	–	Adrenal cortex imaging
	[^{131}I]-19-Iodocholesterol[14]	–	
	[^{131}I]-NP-59*[14]	–	
Phenylethanol-amine-*N*-methyl-transferase	[^{3}H]-SKF-64139*[15]	3×10^{-9}	Adrenal medulla localisation
Acetylcholin-esterase	[^{125}I]-4-Iodophenyltrimethyl-ammonium salt[16]	–	Potential heart imaging agent
	Decamethonium bromide*[17]	25×10^{-6}	
	[^{32}P]-Diisopropyl fluoro-phosphate[18]	–	Red cell label

*IUPAC names: 17β-Hydroxy-4,4′-17α-trimethyl-androst-5-ene(2,3,d) (Isoxazole); 2-(3-Iodo-4-aminophenyl ethylamine (3-Iodo-SKF-12185); [^{131}I]-19-Iodo-cholest-5(6)-en-3β-ol ([^{131}I]-19-Iodocholesterol); 6β-[^{131}I]-Iodomethyl-19-norcholest-5(10)-en-3β-ol (^{131}I-NO-59); 7,8-Dichloro-1,2,3,4-tetrahydroisoquinoline (SKF-64139); *N,N,N,N′,N′,N′*-Hexamethyl-1,10-decanediaminium dibromide (Decamethonium bromide).

tion in red cells, limiting the dose of the inhibitor, phosphorofluoridic acid bis(1-methylethyl) ester (diisopropyl fluorophosphate) to 0.02 mg/Kg body weight.[7] [^{131}I]-N-[p-[(2,4-diamino-6-pteridylmethyl)amino]-3′-iodobenzoyl]-glutamic acid (3′-iodoaminopterin) is not tumor specific, being taken up by organs (liver and kidneys) with high levels of the target enzyme rather than by the virus-induced dihydrofolate reductase in neoplastic cells.[6] In contrast to 2-methyl-1,2-di-3-pyridyl-1-propanol ([^{3}H]-metyrapol), the radioiodide analogue [^{125}I]-5′-iodometyrapol is not taken up effectively by the adrenal cortex, due to lowered affinity for the cytochrome P-450 component of 11β-hydroxylase.[8] The latter system also demonstrates the problem of accessibility; the target enzyme being present in the inner mitochondrial membrane.[9]

On the basis of selective localisation in target tissues, the following enzymes may also offer themselves as further candidates for this enzyme-inhibitor approach: histaminease and ornithine decarboxylase for detecting/imaging malignancy,[4] acyl-CoA dehydrogenase and enoyl-CoA isomerase for imaging the myocardium;[5] α-amylase for imaging the pancreas, and acid phosphatase for imaging the prostate,[3] angiotensin converting enzyme for imaging the lungs;[10] and carbonic anhydrase for imaging the blood pool; this approach is discussed in more detail (*vide infra*).

Red Blood Cell Labelling

Radiolabelled rbc have many diagnostic applications in nuclear medicine including blood pool dynamics, the analysis of cardiovascular disease, and the detection of hemangiomas and gastrointestinal bleeding.[19-23] These red cells are also amenable to the enzyme-inhibitor approach and may serve as the paradigm for all other studies since these cells contain 90% of the body's complement of the enzyme carbonic anhydrase.[24] The system is ideal since the enzyme is present[25] at a high concentration [130–200 μmol dm^{-3}] and aromatic sulphonamide inhibitors are known[26] which bind tightly (K_D < 1 μmol dm^{-3}).

Current labelling methods

The current red cell labelling protocols, which rely upon a non-specific approach, have been splendidly reviewed.[23] The cells may be labelled for short term (^{99m}Tc), intermediate (^{67}Ga, ^{111}In, ^{113}In) or long term studies (^{51}Cr, ^{59}Fe, ^{75}Se); these radiometals bind mainly to haemoglobin. In addition to random, indiscriminate labelling of other blood cells, the following criticisms may also be levied:

1. *In Vivo* labelling with ^{99m}Tc requires two injections, involving pre-administration of a stannous reducing agent followed by [^{99m}Tc]-pertechnetate some 30 min later. Unfortunately, the labelling rate is relatively slow and is accompanied by an initial (20 min) extra-vascular distribution of a proportion of the radionuclide.[27] This problem can be circumvented by *in vitro* labelling methods[19,23,28,29] but this results in a more complex protocol and an additional radiation hazard to the radiopharmacist.[30]
2. While the decay characteristics make ^{99m}Tc most suitable for short term studies, its use may be restricted by gradual elution from the red cells [$t_{1/2} \sim 29$ h][31] and by its short decay half-life (6.0 h). Other radionuclides also suffer from elution problems, hence better intermediate (2–4 days) and long-lived (30 days) labels are needed.
3. The current method for determining the red cell life-span, involving [^{51}Cr]-sodium chromate, is far from ideal. Variable label elution is a significant problem and interpretation of the data can be particularly difficult, especially in haemolytic states.[7]

4. The efficiency of labelling is adversely modified in various diseased states and adversely affected by a number of drugs, including antibiotics, anticonvulsants, antihypertensives, antimicrobials and anti-inflammatory agents.[23,32–35] A well defined, selective labelling approach such as that afforded by a specific enzyme inhibitor should allow logical resolution of the above difficulties.

Enzyme-inhibitor approach

The target enzyme The zinc metalloenzyme carbonic anhydrase, (CA, carbonate hydrolase, EC 4.2.1.1) presents itself as a near ideal target within red cells. It is

TABLE II

Rat CA activity (per gram wet tissue) and CA isozyme distribution in some mammalian tissues and cells[37,41,42,43,44]

Tissue		Enzyme acitivity† (per gram wet tissue)	Detection of: CA I	CA II	CA III
Blood		2400 (rbc)	(+)	(+)	(+)#
Bone and cartilage		-	(0)	(+)	(−)
Muscle:	White skeletal	< 6	(−)	(+)	(−)
	Red skeletal	-	(−)	(−)	(+)#
Adipose cells (white fat cells)		-			
Brain		60 (Glial cells)	(0)	(+)	(−)
Eye:	Lens	50	(0)	(+)	(−)†
	Retina	450	(0)	(+)	(−)#
Heart		Trace (No Hb)	(−)	(−)	(−)
Lungs		30	(0)	(+)	(−)#
Liver		20	(−)	(+)	(−)#
Spleen		-	(−)	(−)	(−)
Pancreas		10	(0)	(+)	(−)#
Kidneys:		248			
	Renal cortex	-	(0)	(+)	(−)
	Renal medulla	-	(−)	(+)	(−)#
Gall bladder		-	(+)	(+)	(−)
Stomach:	Whole	434			
	Parietal cells	7200	(0)	(+)	(−)
Intestine:	Duodenum	77	(−)	(−)	(−)
	Jejunum (mucosa)	26	(0)	(+)	(−)
	Ileum (mucosa)	19	(0)	(+)	(−)
	Caecum (mucosa)	300	(+)	(+)	(−)
	Appendix (epithelium)	-	(+)	(+)	(−)
	Colon (columnar cells)	345	(+)	(0)	(−)#
	Rectum	-	(−)	(−)	(−)
Salivary glands		-	(+)	(+)	(−)†
Sweat glands		-	(+)	(0)	(−)#
Thyroid gland		< 30	(−)	(−)	(−)
Prostate gland:	Posterior	2400	(−)	(−)	(−)
	Ventral	28	(−)	(−)	(−)
Breast		116	(−)	(−)	(−)
Testes		-	(−)	(−)	(−)
Uterus		0	(−)	(−)	(−)
Pregnant uterus		130	(−)	(−)	(−)

Soluble CA isozymes were identified by immunohistochemical or purification procedures; isozyme detected (+), isozyme not detected or trace levels attributed to red cell contamination (0), and not tested (−). The CA I and CA III isozymes are the low activity type and CA II is the high activity isozyme found in rbc. #Refers to data for human tissue, and †refers specifically to rat tissue. Hb represents Haemoglobin.

selectively localised at a high concentration within the cytosol, and is potently inhibited by aromatic sulphonamides. All known biochemical functions of the enzyme are related to its remarkably efficient catalysis of the reversible hydration of carbon dioxide.[26,36] The human red blood cells contain four distinct CA isozymes, HCA, I_1, HCA I, HCA II and HCA III with relative abundance 5, 83, 12 and 1%, respectively.[37–39] These isozymes differ in their specific catalytic activities; for example HCA I is a 'low activity' variant, whereas HCA II is a 'high activity' type and is one of the most efficient of all known enzymes.[26,36,37]

Distribution of carbonic anhydrase In humans, the major portion of the body's complement of CA is found in rbc, with less than 10% of the total enzyme being present in the various tissues of the body.[24,25,28] Mammalian blood contains 1–2 g/dm^3 of CA,[39] the enzyme being one of the major protein components of rbc after haemoglobin.[40] The above CA isozymes have also been found in mammalian tissues, but in general, at a lower concentration (Table II). In addition, a membrane bound isozyme, HCA IV, has been identified using immunohistochemical techniques.[45] More recently, a total of seven genetically distinct forms of mammalian carbonic anhydrases have been identified; CA V in mitochondria, CA VI in salivary glands (acinar cells) and CA VII (membrane bound) also in salivary glands.[46]

Reversible inhibition The two main human red cell isozymes HCA I and HCA II exhibit broadly similar inhibition as a result of their close primary, secondary and tertiary structures. There are 260 and 259 amino acid residues in HCA I and HCA II isozymes, respectively, with 59% sequence homology. These isozymes are reversibly inhibited by many mono anions (Table III). It is important to note that the chloride and bicarbonate ions normally present in red cells, while not appropriate as carriers in their own right, may compete *in vivo* with the chosen carrier.

The first choice of a carrier would be the aromatic and heterocyclic sulphonamides (RSO_2NH_2) which are highly specific and very potent non-competitive inhibitors of the HCA isozymes;[36,50,51] in some cases binding so tightly to give pseudo-irreversible inhibition.[26,39,52] In contrast to anions, the sulphonamides generally bind more tightly to the HCA II than to the HCA I isozyme (Table IV). The potent sulphonamide

TABLE III
Apparent dissociation constants for monovalent anions and the two predominant red cell CA isozymes

Anion	Apparent dissociation constant† (K'_D) (mol dm^{-3})	
	HCA I	HCA II
I^-	2.0×10^{-3}	3.1×10^{-3}
SCN^-	7.0×10^{-4}	8.0×10^{-4}
Cl^-	5.1×10^{-3}	6.7×10^{-2}
Br^-	2.3×10^{-2}	2.7×10^{-2}
CH_3COO^-	2.6×10^{-2}	3.4×10^{-2}
HCO_3^-	1.2×10^{-2} #	7.0×10^{-2} #
F^-	0.4	

†Measured[47] from inhibition of the catalysed hydrolysis of *p*-nitrophenyl acetate at pH 7.3 for HCA I and pH 6.8 for HCA II, 25°C. #Measured in 7 mmol dm^{-3} barbital buffer[48]. Recent results[49] suggest a slight discrepancy for the above K'_D values; by a factor of 10 fold for HCA I–SCN^- and 6 fold for HCA I–Cl^-, but these were done at a pH of 6.8.

TABLE IV
Apparent dissociation (K'_D) of the complexes between human carbonic anhydrases and various sulphonamides

Sulphonamide	K'_D(mol dm^{-3})	
	HCA I	HCA II
Benzenesulphonamide	–	1.5×10^{-6}**
Sulphanilamide*	5.0×10^{-5}#	2.0×10^{-6}#
4-Nitrobenzenesulphonamide	1.7×10^{-7}**	6.3×10^{-8}**
4-Methylbenzensulphonamide	–	5.1×10^{-7}**
4-(Benzenesulphonyl)benzene-sulphonamide	6.4×10^{-8}†¥	
4-(Salicyl-5-azo)benzene-sulphonamide	–	2.9×10^{-9}**
2-Nitrothiophene-5-sulphonamide	9.5×10^{-9}**	$< 1.0 \times 10^{-8}$**
2-(4-Fluorobenzenesulphonyl-thiophene-5-sulphonamide	9.0×10^{-9}†¥	
Acetazolamide*	2.0×10^{-7}#	1.0×10^{-8}#
Methazolamide*	1.0×10^{-8}#	1.0×10^{-8}#
Ethoxzolamide*	2.0×10^{-8}#	2.0×10^{-8}#

*IUPAC names: 4-Aminobenzenesulphonamide (Sulphanilamide); *N*-[5-(Aminosulphonyl)-1,3,4-thiadiazol-2-yl]acetamide (Acetazolamide); *N*-[5-(Aminosulphonyl)-3-methyl-1,3,4-tridiazol-2(3H)ylidene]acetamide (Methazolamide); 6-Ethoxy-2-benzothiazolesulphonamide (Ethoxzolamide). ¥Refers to the use of a mixture of HCA I and HCA II isozymes. **Refers to the measurements done[53] at 25°C, pH 6.5, †refers to measurements done[54] at 0°C and # refers to measurements done[38,55] at 0°C, pH 7.0–7.5.

inhibition is due to binding interactions at the active-site cavity of the isozymes. Both the HCA I and the HCA II isozymes are ellipsoidal (Figure 1), with the active-site being located in a conical cavity which is 1.5 nm wide at the entrance and 1.5 nm deep.[56] The sulphonamido group is bound to the essential zinc(ii) ion of the isozymes through an ionised nitrogen atom, with an additional long bond through an oxygen atom. In addition, there are other interactions between the sulphonamide molecule and the isozymes such as van der Waal's contact with Val-121, Ile-91 and Phe-91 of HCA II and with Ala-121 and Phe-91 of HCA I.[36] Such hydrophobic interactions have been confirmed for acetazolamide by computer model simulation studies.[57] However, recent X-ray crystallographic data[58] rules out any binding of the sulphonamido oxygen atoms to the zinc ion of the HCA II isozyme. Also, the van der Waal's interactions of the acetazolamide molecule with the HCA II isozyme have been reassigned to Val-121, Gln-92, and Leu-198, but there seems to be uncertainty for the interactions of the acetylamido moiety.

Although an unsubstituted sulphonamide group linked to a conjugated ring is an absolute requirement for potent inhibition, substantial modification and extension of the ring are possible while still retaining or even enhancing the characteristic high affinity (cf Table IV.). This represents yet another attractive character with regard to the choice of this type of inhibitor as a radiolabel carrier.

Irreversible inhibition

While very tight binding may provide pseudo-irreversible inhibition, there may be certain circumstances, such as in long term red cell studies when truly irreversible binding is required. Irreversible inhibitors of the active-site-directed type[59] are known

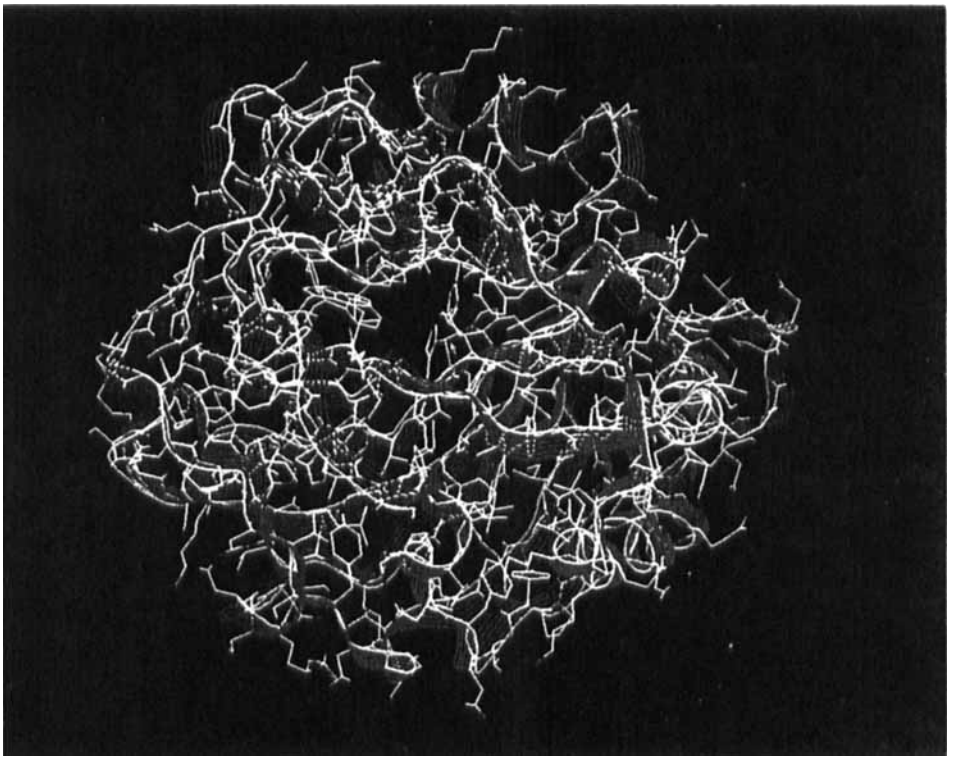


FIGURE 1 The IRIS Workstation Silicon Graphics Computer drawing of the ellipsoidal HCA I isozyme as derived from the Cambridge Protein Data Bank [2CAB. Carbonic anhydrase B (Human), K. Kannan, 10/83]. The computer was run on the program called Quanta, provided by Polygen. The 3-dimensional amino acid sequence together with the strands of the β-structure of the target isozyme are presented. The hole in the middle of the molecule is the active-site cavity where the essential zinc(ii) ion (as indicated by the arrow) is coordinated to the histidine residues 94, 96 and 119. (See colour plate at back of issue).

for both the HCA I and HCA II isozymes (Tables V and VI). These inhibitors initially bind reversibly at the active-site, followed by a time-dependent alkylation of a specific active-site amino acid residue. The initial reversible binding should afford specificity for the inhibitor but this may in fact depend on the reactivity of the alkylating group. If non-specific alkylation is rapid relative to targeting, selectivity would be lost. For *in vivo* application, a radiolabelled irreversible inhibitor would therefore need to exhibit rapid uptake into red cells with immediate tight binding to the target enzyme, followed by a slower irreversible alkylation step.

The carrier molecule Various sulphonamides, evaluated as carbonic anhydrase inhibitors, have already found clinical application as diuretics,[67] cerebral vasodilators,[54,68,69] anticonvulsants,[70,71,72] anti-glaucoma agents[38,73] and in the treatment of hydrocephalus.[74] Accompanying studies have demonstrated that sulphonamides such as acetazolamide and methazolamide can reach the target enzyme within red blood cells and bind tightly to it *in vitro* and *in vivo*.[24,74-77] In addition, the sulphonamides appear to be very selective for the target enzyme within the red cell and have little or

TABLE V
Some irreversible inhibitors of the HCA I isozyme

HCA I Inhibition	K'_D(mol dm^{-3})†	Irreversible	Inactivation
		$t_{1/2}$	Modified residue
Bromoacetate	3.8×10^{-3}	1 h	3'N-His 200
Bromopyruvate	3.1×10^{-3}	7 min	3'N-His 200
Iodoacetate	1.5×10^{-3}	0.6 h	3'N-His 200
Bromoacetazolamide*	1.3×10^{-6}	very slow	3'N-His 64
N-(Bromoacetyl)acetazolamide*	–	rapid	1'N-His 67
N-(Chloroacetyl)chlorothiazide*	2.0×10^{-9}	16 h	3'N-His 67
N-(Chloroacetyl)cyclothiazide*	1.5×10^{-8}	6 h	3'N-His 67

The apparent dissociation constant of the initial reversible enzyme-inhibitor complex and the half-life ($t_{1/2}$) of the subsequent alkylation of a histidine residue are given[56,60–66]. †Measured at pH 7.6, 25°C. The $t_{1/2}$ is the half-time for 50% of the enzyme activity to be irreversibly inactivated. One equivalent of the inhibitor has been used in the inactivation studies, except for bromopyruvate and iodoacetate (20 fold molar excess) and *N*-(bromoacetyl)acetazolamide (10 fold molar excess, pH 7.2). *IUPAC names: *N*-[5-(Aminosulphonyl)-1,3,4-thiadiazol-2-yl]bromoacetamide (Bromoacetazolamide); 5-[*N*(Bromoacetyl)aminosulphonyl]-1,3,4-thiadiazol-2-yl]acetamide (*N*(-Bromoacetyl)acetazolamide); 6-Chloro-1,2,4-benzothiadiazine-7-*N*-chloro acetylaminosulphonyl 1,1-dioxide (*N*-(Chloroacetyl)chlorothiazide); 3-Bicyclo-[2.2.1]hept-5-en-2-yl-6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-N-chloroacetylaminosulphonyl 1,1-dioxide (*N*-(Chloro acetyl)cyclothiazide).

no effect on other blood enzymes such as cytochrome oxidase, catalase and peroxidase.[50]

Limitations on carrier sulphonamides Unlike other red blood cell enzymes such as cholinesterase and glucose-6-phosphate dehydrogenase, carbonic anhydrase does not undergo modification during the life-span of the cell in human adults.[78,79] A carrier sulphonamide should therefore bind with equal avidity to the target enzyme in young and old cells. Although the mean adult level[24,25] of the isozymes in blood [HCA I: 136–165 μmol dm^{-3}; HCA II: 17–20 μmol dm^{-3} is not found until the age of sixteen,[80] it is already 20% of this level at birth.[41] These levels represent a 500-fold excess over that which is apparently required for efficient catalysis,[42] so that fractional saturation by a radiolabelled inhibitor is not a concern, even to the neonate.

This situation may be complicated however, in patients undergoing sulphonamide medication (although in general the bacteriostatic sulphonamides, which do not bind

TABLE VI
Some irreversible inhibitors of the HCA II isozyme

HCA II Inhibition	K'_D(mol dm^{-3})†	Irreversible	Inactivation
		$t_{1/2}$	Modified residue
Bromopyruvate	2.4×10^{-2}	slow	3'N-His 64
Bromoacetate#	2.3×10^{-1}	slow	3'N-His 64
Iodoacetate#	–	slow	3'N-His 64
Bromoacetazolamide	1.1×10^{-6}	rapid	3'N-His 64
N-(Bromoacetyl)acetazolamide	–	–	3'N-His 64

The apparent dissociation constant of the initial reversible enzyme-inhibitor complex and the half-life ($t_{1/2}$) of the subsequent alkylation of a histidine residue are given[56,63,65,66]. †Similar conditions apply as for Table V, except for bromopyruvate which was carried out at pH 6.8. #Inhibitor also causes non-specific modification of other amino acid residues (not shown).

to carbonic anhydrase, present no problem). Competition between the carrier and the drug could vitiate the application of this approach. Also, the use of sulphonamide carriers could result in an allergic reaction, but their relatively low molecular weight would make them very poor antigens for an antibody response and thus minimise any sensitive reaction. The side reactions described in the early sulphonamide era[81] are unlikely to result when using these inhibitors as carriers, since they will be present at a low concentration. However, reports of haemolysis accompanying the administration of, for example, sulphanilamide, must be borne in mind.

The Ideal Red Cell Label On the basis of the above discussion it is possible to outline the characteristics of the ideal sulphonamide for red cell labelling:

1. It should have a high affinity for carbonic anhydrase. While the target enzyme concentration is high in red cells ($\sim 10^{-4}$ mol dm^{-3}), the competition afforded by mono anions such as chloride and bicarbonate, dictates that the intrinsic affinity of the carrier should be greater than 10^7 mol^{-1} dm^3, to ensure a suitable localisation index.[82] In addition, high affinity is required to prevent any significant washout or exchange with extravascular CA targets.
2. The sulphonamide should be sufficiently lipophilic to rapidly penetrate the red cell membrane and so reach the cytosolic target. This suggests that in the simplest case the pK_a of the sulphonamido group should be above neutral.
3. Facile radiolabelling of the carrier should be possible without adversely affecting the above characteristics.
4. A carrier which is non-immunogenic and which has a long shelf-life would also be advantageous.

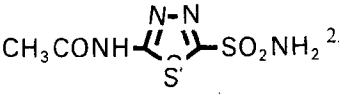
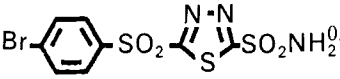
Honda and co-workers reported disappointing red cell labelling results in mice for [^{125}I]-N-(iodacetyl)acetazolamide,[83] a sulphonamide that would appear to possess the above characteristics. However, their choice of labelling the alkyl iodide atom was unfortunate. Like its *N*-chloroacetyl analogue, an irreversible reaction with the target enzyme will result in a loss of the alkyl halogen atom.[60] Moreover, the high reactivity of this alkylating sulphonamide would be non-specific. Therefore, it is not surprising that the reported radioactivity distribution in tissues did not reflect the CA concentration in those organs.

OBJECTIVE DESIGN

The enzyme-inhibitor approach would seem to be ideal for red cell labelling. Both pseudo-irreversible and irreversible sulphonamide inhibitors could offer significant advantages over current methodology for short, intermediate and long-term studies. The next step, the search for an appropriate red cell-specific, sulphonamide carrier becomes a rational design process when the wealth of information about the target enzyme is coupled with the necessary characteristics of the carrier inhibitor.

Benzensulphonamide was chosen as the basic carrier moiety and substituents were introduced at the para position to provide maximum binding to the target enzyme[57,84] (Table VII, **1–5**). Aryl iodide was incorporated to allow radiolabelling via radioiodide exchange. Sulphonamides **2–5** were designed to include an extra aromatic ring since such extended systems seem to enhance binding affinity for the target enzyme.[57] Sulphonamides **4** and **5** were selected on the basis of the potent inhibition provided by analogous aryl thio- and aryl sulphonyl-substituted aromatic and heterocyclic

TABLE VII
Kinetic and physical properties of some sulphonamide inhibitors of carbonic anhydrase

Sulphonamide			Apparent Dissociation Constant ± 1 SD (mol dm^{-3})*			
No	R_1	R_2	HCA I	HCA II	$pK_a^{\dagger}$ (± 0.07)	Log P (± 0.03)
1.	I	H	$1.44 \pm 0.12 \times 10^{-8}$	$1.36 \pm 0.01 \times 10^{-8}$	10.06	0.90
2.	$IC_6H_4NHSO_2$	H	$0.66 \pm 0.33 \times 10^{-7}$	$0.49 \pm 0.06 \times 10^{-8}$	–	0.78
3.	$IC_6H_4SO_2NH$	H	$1.75 \pm 0.03 \times 10^{-7}$	$2.36 \pm 0.48 \times 10^{-7}$	7.40	−0.66
4.	IC_6H_4S	H	$1.56 \pm 0.56 \times 10^{-8}$	$0.43 \pm 0.32 \times 10^{-8}$	–	1.13
5.	$IC_6H_4SO_2$	H	$1.81 \pm 0.45 \times 10^{-8}$	$1.35 \pm 1.00 \times 10^{-9}$	9.10[¥]	0.10
6.	CH_3CONH	H	$1.18 \pm 0.20 \times 10^{-6}$	$2.03 \pm 0.39 \times 10^{-7}$	9.71	−0.09
7.	$ClCH_2CONH$	H	$1.14 \pm 0.04 \times 10^{-7}$	$0.97 \pm 0.01 \times 10^{-7}$	–	–
8.	$ClCH_2CONH$	I	$0.94 \pm 0.21 \times 10^{-7}$	$1.15 \pm 0.15 \times 10^{-7}$	–	–
9.	$BrCH_2CONH$	H	$1.07 \pm 0.02 \times 10^{-7}$	$0.91 \pm 0.09 \times 10^{-7}$	–	–
10.	ICH_2CONH	H	$0.40 \pm 0.09 \times 10^{-7}$	$0.19 \pm 0.01 \times 10^{-7}$	–	–
11.	H	$ClCH_2CONH$	$3.47 \pm 0.57 \times 10^{-6}$	–	–	–
12.	H	ICH_2CONH	$2.55 \pm 0.23 \times 10^{-6}$	–	–	–
13.	I	ICH_2CONH	$1.49 \pm 0.58 \times 10^{-6}$	$0.70 \pm 0.20 \times 10^{-7}$	> 10	0.28
14.	I	CH_3CONH	$0.45 \pm 0.06 \times 10^{-6}$	$0.71 \pm 0.05 \times 10^{-7}$	–	–
15.			$2.73 \pm 0.29 \times 10^{-7}$	$1.55 \pm 0.40 \times 10^{-8}$	7.46	−0.25[©]
16.			$0.21 \pm 0.30 \times 10^{-8}$	$0.25 \pm 0.66 \times 10^{-9}$	8.29[¥]	1.81[¥]

The apparent dissociation constant values were obtained from a least square regression procedure as ± 1 standard deviation (SD). For synthesis of sulphonamides **1–14,** see Experimental Section. *Measured at 25.0 ± 0.5°C in a Tris-Sulphate buffer (10 mmol dm^{-3}, pH = 8.00, μ = 0.1 mol dm^{-3}). *p*-Nitrophenyl acetate was used as the substrate and non-competitive inhibition was assumed. †Measured at 23.0 ± 0.5°C in aqueous ethanol (up to 40% v/v); μ = 0.1 mol dm^{-3}. ¥From the literature[54]; for sulphonamide **5,** data refers to an analogous compound without an iodide atom and for sulphonamide **16,** the pK_a data refers to the 4-fluoro analogue. Log P values are from chloroform/pbs partitioning, with the exception of sulphonamides **15** and **16** which refer to octanol/pbs partitioning; ©refers to data from the literature[68].

sulphonamides[54] such as **16** (Table VII). These choices were also prompted by the apparent long retention in red cells ($t_{1/2}$ = 120 days in man) of the anticonvulsant CA inhibitor 2-amino-4-(phenylsulphonyl)benzenesulphonamide.[85]

Active-site-directed, irreversible inactivation of the target enzyme by sulphonamide inhibitors such as bromoacetazolamide involves an initial reversible binding step, followed by a time-dependent covalent modification of an adjacent active-site amino acid residue.[59,61] Such irreversible inactivation could be effected by a benzenesulphonamide derivative carrying a reactive haloacetyl or a haloacetamido group. The latter group was chosen for ease of synthesis (Sulphonamides **7–13,** Table VII). It has been reported that 4-chloroacetamidobenzenesulphonamide (**7**) reversibly inhibits the HCA I isozyme ($K'_D = 2.5 \times 10^{-6}$ mol dm^{-3} at pH 7.6) but causes no irreversible inactivation of the isozyme, probably due to unfavourable orientation of the reactive group with respect to the modifiable groups on the enzyme.[60] The effect of this sulphonamide on HCA II has not been reported, so sulphonamide **7** was prepared. In

addition, the more reactive bromo (**9**) and iodo (**10**) analogues were synthesised to assess whether isozyme inactivation could simply be a kinetic phenomenon.

Two derivatives of the potent reversible CA inhibitor acetazolamide (**15**), namely *N*-(bromoacetyl)acetazolamide and bromoacetazolamide seem to specifically, irreversibly inactivate the HCA I and HCA II isozymes, respectively.[56] This difference reflects the subtle orientation difference of the modifiable group in the two HCA isozymes. So, in addition to the above para-substituted benzenesulphonamides, meta-substituted sulphonamides **11–14** which gave a closer structural analogue to the above 'inactivating' sulphonamides, were synthesised (Table VII).

In the following sections of this paper, we describe the synthesis of the above sulphonamides together with inhibition data for the two human carbonic anydrase isozymes, HCA I and HCA II. The lipophilicity (Log *P*) and ionization constant (pK_a) of some of the sulphonamides are also given. These results would allow a more rational choice of potential carriers for further red cell labelling studies.

EXPERIMENTAL SECTION

Instrumentation and Methods

Melting points (mp) were determined on an Electrothermal or Gallenkamp capillary apparatus and are uncorrected. Infrared spectra (IR) were recorded on a Perkin Elmer Spectrometer-599 or -589 B, using a Nujol mull method, with sodium chloride plates ($4000–550\,cm^{-1}$). Proton nuclear magnetic resonance (^1H NMR) spectra were recorded at 60 MHz on Perkin Elmer R12 or R243 spectrometers, and at 100 MHz on a Varian XL 100 spectrometer. All samples were dissolved in a suitable deuterated solvent, with δ-values reported downfield from TMS. A ^{13}C NMR spectrum was also recorded on the Varian spectrometer with complete proton spin-decoupling and a chemical shift range of 0–200 ppm (δ-values). The δ-values of the carbon atoms are given together with those calculated using empirical parameters given in the literature.[86] Mass spectra (MS) were obtained by electron impact at 70 eV on a Kratos MS-30 Spectrometer. Ultraviolet spectra (UV) were obtained on a thermostated Double-Beam UV-190 Shimadzu Spectrometer. Elemental analyses were carried out on an F and M analyser, Model 185 and the values reported are within $\pm 0.4\%$ of the theoretical values. The purity of the compounds were analyzed by thin layer chromatography (tlc) on Polygram Sil G/UV 254 precoated silica gel plastic sheets, obtained from Camlab Cambridge; spots were visualised by UV fluorescence and/or via iodine vapour.

Partition Coefficient measurements were carried out using the solvent system chloroform/phosphate buffered saline (pbs). Results are expressed as Log *P*, where *P* represents the ratio of the concentration in the organic layer to that in the aqueous layer. The acidity constants (pK_a) were determined by a potentiometric titration method, as outlined in the literature.[87] The pK_a value was determined graphically from the titration curve or via computer analysis using the program PKAS adapted for the BBC microcomputer from the literature.[88]

Preparation of Solutions and Reagents

All solutions were made in double distilled water. The pH of buffered solutions was measured using a PTI-15 Digital pH meter that had been calibrated with two standard

buffers. The pbs (1 L, pH 7.4) was made with sodium chloride (7.02 g), potassium dihydrogen orthophosphate (0.79 g) and disodium hydrogen phosphate (3.45 g); Tris-Chloride (5.0 mmol dm^{-3}) plus ethylenediaminetetraacetic acid (EDTA) (1 mmol dm^{-3}, 1 L, pH 7.5) solution was made with tris(hydroxymethyl)aminomethane (Tris) (0.60 g) and disodium salt of EDTA (0.38 g), the pH adjusted with 2N HCl; Tris-Sulphate (10 mmol dm^{-3}, 1 L, pH 8.0) was made with Tris (1.21 g) and sodium sulphate (4.39 g), the pH adjusted with 2N H_2SO_4; Tris-Sulphate (5.9 mmol dm^{-3}, 8.5 L, pH 8.5) was made with Tris (6.05 g), the pH adjusted with 2N H_2SO_4; Tris-Chloride (0.05 mol dm^{-3}, 1 L, pH 8.7) was made with Tris (6.06 g), the pH being adjusted with 2N HCl.

Anhydrous acetone, dry pyridine, dry tetrahydrofuran (THF), pure light petroleum ether (bp 40–60°C) and pure analar chloroform were obtined by standard literature methods.[89,90] Raney nickel was prepared from a nickel-aluminium alloy by the selective precipitation of aluminium hydroxide as described in the literature.[90] Cuprous iodide was also prepared by a literature method.[90] *N*-[5-(aminosulphonyl)-1,3,4-thiadiazol-2-yl]acetamide (acetazolamide) from Koch-Light Lab. was recrystallised from boiling water, and from absolute alcohol and the dried *in vacuo,* mp 264–265°C (d), (lit. 260–261°C).[91] p-Iodobenzenesulphonyl chloride (Aldrich) was recrystallised from analar chloroform and pet. ether (1: 5.5, v/v), mp 82–83°C (lit. 84°C).[92]

Iodine monochloride (ICI) was prepared as follows: potassium iodate (4.8 g, 0.022 mol) was added to a solution of potassium iodide (7.2 g, 0.043 mol) in water (12 mL). The mixture was shaken and concentrated hydrochloric acid (15 mL) added. The resulting dark purple suspension of ICI was stirred for 2 h before use.

4-Chlorosulphonylbenzenesulphonamide was prepared from sulphanilamide according to a literature method.[93] 3-Iodo-4-aminobenzenesulphonamide, recrystallised from 10% aqueous absolute ethanol, mp 180–184°C (d), (lit. 179–180° C)[94] was made by a literature method.[95] 3-Nitrobenzenesulphonamide was prepared from nitrobenzene by a literature method.[96] The crude product was recrystallised from hot water to give a pure white product, mp 167–169°C, (lit. 162–163°C). NMR and IR data were consistent with the structure of the sulphonamide. 3-Amino-4-iodobenzenesulphonamide was prepared from 3-aminobenzenesulphonamide by a literature method. The crude product was recrystallised from hot water, mp 168.5–170.5°C, (lit. 170° C).[97]

Synthetic Chemical Methods

p-Iodobenzenesulphonamide (**1**). Anhydrous pyridine (6 mL; 0.074 mol) was added to *p*-iodobenzenesulphonyl chloride (10.3 g; 0.034 mol) in anhydrous acetone (40 mL). The mixture was stirred for 1 h at room temperature, then bubbled with dry ammonia gas for 3 h and left at room temperature for 60 h. The resulting red, alkaline solution was neutralised with 2N hydrochloric acid and immediately an off-white precipitate formed. The product was filtered, washed quickly with chilled chloroform and recrystallised twice from boiling water to give white crystals of **1** (6.4 g, 67%), mp 186–189°C, (lit. 190° C;[92] different method). ^{1}H NMR (100 MHz, acetone): 7.84 (q, 4H, Φ), 6.68 (bs, 2H, SO_2NH_2); ^{13}C NMR (acetone): 144.9 (s, C_1, δ^{calc} 145), 128.1 (s, C_2, δ^{calc} 128.7), 138.9 (s, C_3, δ^{calc} 139.3), 99.1 (s, C_4, δ^{calc} 100); IR: 3360, 3259, 1295, 1158, 1092 cm^{-1}. Found: C, 25.5 H, 2.1; N, 4.8. $C_6H_6INO_2S$ requires; C, 25.4; H, 2.1; N, 4.9%. MS, m/z 283 (M^+).

4-[(4-Iodophenyl)-N'-aminosulphonyl]benzenesulphonamide (2). Iodoaniline (0.94 g; 4.29 mmol) in anhydrous pyridine (600 μL; 7.43 mmol) was added to 4-chlorosulphonylbenzenesulphonamide (0.80 g; 3.13 mmol) in anhydrous acetone (6 mL). The reddish orange solution was left to stand at room temperature for 2 days. On neutralising with 2N hydrochloric acid and diluting with water (50 mL), a white precipitate formed. The product was filtered, washed with water and chilled chloroform, and recrystallised from aqueous acetone to give **2** (1.36 g, 99%) mp 249–251°C (d). ^{1}H NMR (60 MHz, DMSO): 7.82 (s, 4H, ΦSO$_2$), 7.36 (bs, 2H, SO$_2$NH$_2$), 7.12 (q, 4H, IΦ), 4.78 (bs, 1H, ΦN*H*SO$_2$); IR: 3364, 3252, 1339, 1165, 1092, 898 cm^{-1}. Found: C, 33.3; H, 2.7; N, 6.0. $C_{12}H_{11}IN_2O_4S_2$ requires; C, 32.9; H, 2.5; N, 6.4%. MS, m/z 438 (M^+).

4-[(4-Iodophenyl)sulphonamido]benzenesulphonamide (3). *p*-Iodobenzenesulphonyl chloride (0.51 g; 5 mmol) in anhydrous pyridine (600 μL; 7.43 mmol) was added to sulphanilamide (0.86 g; 5 mmol) in anhydrous acetone (5 mL). The reaction mixture was worked up as in the preparation of **2**. The precipitate was recrystallised thrice from aqueous acetone and then once from ethyl acetate/pet. ether (1:1, v/v) to give pure **3** (1.5 g, 70%), mp 221–222.5°C. IR: 3364, 3260, 1338, 1160, 1088, 910 cm^{-1}; Found: C, 32.7; H, 2.3; N, 6.6. C_{12}, $H_{11}IN_2O_4S_2$ requires C, 32.9; H, 2.5; N, 6.4%. MS, m/z 438 (M^+).

4-[(4-Iodophenyl)thio]benesulphonamide (4). An aqueous solution (5 mL) of sodium hydroxide (0.51 g; 12.7 mmol) was added to a DMF solution (25 mL) of 4-aminobenzenethiol (1.60 g, 12.8 mmol). To this was added a DMF solution (30 mL) of sulphonamide **1** (3.60 g; 12.7 mmol). The reaction mixture was refluxed for 1 h under nitrogen, and then the solvent evaporated under reduced pressure. On adding chilled water (150 mL), an off-white pricipitate was obtained. This was collected by filtration, washed thoroughly with chilled water, dried and recyrstallised from ethyl acetate/pet. ether (1:1, v/v) to give pure 4-[(4-aminophenyl)thio]benzenesulphonamide, (4-ATBS, 2.46 g; 46%) mp 182–185°C. ^{1}H NMR (acetone): 7.93–6.64 (qq, 8H, ΦSΦ), 6.44 (bs, 2H, SO$_2$NH$_2$), 5.09 (bs, 2H, NH$_2$Φ); IR: 3439, 3380, 3352, 1307, 1160, 1150, 935 cm^{-1}; MS, m/z 280 (M^+).

4-ATBS (1.20 g; 4.28 mmol) was dissolved in a solution of concentrated hydrochloric acid (10 mL) and water (10 mL). The mixture was mechanically stirred for 1 h at 0°C, before an aqueous solution of sodium nitrite (0.33 g; 4.38 mmol) was added very slowly whilst maintaining the temperature at 0–5°C. The mixture was then stirred for a further hour. An aqueous mixture of potassium iodide (0.71 g; 4.28 mmol) and cuprous iodide (0.41 g; 2.15 mmol) was added at 0–5°C. The mixture was left overnight at room temperature. It was then extracted with ethyl acetate (5 × 200 mL), the combined extracts dried (Mg SO$_4$) and concentrated to a small volume. On adding pet. ether, an off-white precipitate of **4** (1.15 g; 69%) was obtained. Recrystallisation from aqueous ethyl acetate and from benzene/pet. ether (1:1, v/v) gave a pale white product, mp 150–153°C. ^{1}H NMR (100 MHz, DMSO): 7.78 (q, 4H, IΦ), 7.38 (q, 4H, SO$_2$Φ), 7.30 (s, 2H, SO$_2$NH$_2$); IR: 3600, 3538, 3300, 1322, 1160, 1105, 927 cm^{-1}. Found: C, 37.1; H, 2.4; N, 3.6. $C_{12}H_{10}INO_2S_2$ requires; C, 36.8; H, 2.5; N, 3.6%. MS, m/z 391 (M^+).

4-[(4-Iodophenyl)sulphonyl]benzenesulphonamide (5). The sulphonamide **4** (200 mg; 0.51 mmol) was dissolved in acetic acid (15 mL) to which hydrogen peroxide

(1 mL; 8.8 mmol) was added. The mixture was heated on a stream bath for 1 h. Water was added to the hot solution until crystallisation commenced, and then allowed to cool. The white product was filtered, washed with water and recrystallised thrice from ethyl acetate/pet. ether to give **5** (0.19 g, 88%), mp 179–183°C. IR: 3510, 3370, 3278, 1328, 1162, 1110, 905 cm^{-1}; MS, m/z 423 (M^+). TLC in the solvent system, chloroform-methanol-benzene (2:1:1, v/v/v) showed an intense spot at R_f = 0.70 and a faint spot at R_f = 0.75; the latter corresponding to unreacted sulphonamide **4**. This slightly contaminated compound was used without further purification for enzyme inhibition studies.

*p-Acetamidobenzenesulphonamide (**6**).* Acetic anhydride (1.18 g; 11.6 mmol) was added to a solution of sulphanilamide (2.0 g; 11.6 mmol) in acetone, and refluxed for 45 min. The mixture was cooled and then concentrated to a small volume. On adding water (50 mL), a white precipitate formed. This was recrystallized from boiling water and dried to give **6** (1.22 g; 50%), mp 223-225.5°C, (lit. 219–220° C).[98] ^1H NMR (DMSO): 7.70 (s, 4H, Φ), 7.18 (bs, 2H, SO_2NH_2), 3.33 (bs, 1H, CONH), 2.08 (s, 3H, CH_3CO); IR: 3374, 3292, 3214, 1660, 1333, 1160, 1098, 908 cm^{-1}; MS, m/z 214 (M^+).

*4-(Chloroacetamido)benzenesulphonamide (**7**).* Chloroacetic anhydride (4.22 g; 0.025 mol) was added to a diethyl ether/acetone solution (180 mL; 2:9, v/v) of sulphanilamide (5.0 g; 0.029 mol). The mixture was refluxed for 2.5 h. The hot solution was filtered and the solvent concentrated to a small volume. On adding water (50 mL), a white precipitate formed. This was recrystallized from boiling water and dried to give pure **7** (4.0 g; 56%), mp 225–227°C. ^1H NMR (DMSO): 7.75 (s, 4H, Φ), 7.22 (bs, 2H, SO_2NH_2), 4.27 (s, 2H, $ClCH_2CO$), 3.44 (s, 1H, CONH); IR: 3326, 3216, 1690, 1320, 1160, 1098, 905 cm^{-1}; MS, m/z 248 (M^+).

*3-Iodo-4-(chloroacetamido)benzenesulphonmide (**8**).* 3-Iodo-4-aminobenzenesulphonamide (0.15 g; 1.71 mmol) was added to a solution of chloroacetic anhydride (0.35 g; 2.05 mmol) in acetone (10 mL). The mixture was refluxed for 1 h and then cooled. On adding water (100 mL), an off-white precipitate formed. This was washed with chilled diethyl ether and recrystallised from aqueous ethanol (100 mL, 5:1, v/v) to give sulphonamide **8** (0.39 g; 61%), mp 184–186°C. ^1H NMR (100 MHz, DMSO): 8.29 (d, J_{26} = 2.0 Hz, 1H, $H_2Φ$), 7.86 (dd, J_{65} = 8.1 Hz, J_{62} = 2.0 Hz, 1H, $H_6Φ$), 7.73 (d, J_{56} = 8.1 Hz, 1H, $H_5Φ$), 7.49 (bs, 2H, SO_2NH_2), 4.41 (s, 2H, $ClCH_2CO$), 3.34 (s, 1H, CONH); IR: 3320, 3300, 3210, 1680, 1314, 1160, 1104, 900 cm^{-1}; Found: C, 26.0; H, 2.0; N, 7.6. $C_8H_8N_2O_3ClI$ requires: C, 25.6; H, 2.1; N, 7.5%.

*4-(Bromoacetamido)benzenesulphonamide (**9**).* The sulphonamide **7** (0.6 g; 2.41 mmol) was dissolved in acetone (25 mL) to which sodium bromide (0.26 g; 2.53 mmol) was added. The mixture was refluxed for 1.3 h, cooled and concentrated under reduced pressure. On adding water (50 mL), a white precipitate was obtained. Recrystallisation from hot water gave pure **9** (0.26 g; 36%), mp 226–228.5°C (d). ^1H NMR (DMSO): 7.74 (s, 4H, Φ), 7.26 (bs, 2H, SO_2NH_2), 4.28 (s, 2H, $BrCH_2CO$), 3.31 (s, 1H, CONH); IR: 3426, 3210, 1690, 1320, 1160, 1099, 908 cm^{-1}; MS, m/z 292 (M^+).

*4-(Iodoacetamido)benzenesulphonamide(**10**).* This sulphonamide was prepared by a similar method to that employed in the preparation of sulphonamide **9**, but using sodium iodide (0.40 g; 2.67 mmol) instead of sodium bromide. The product was

recrystallised from hot water to give pure **10** (0.6 g; 70%), mp 230–231.5°C (d). ^{1}H NMR (DMSO): 7.72 (s, 4H, Φ), 7.21 (bs, 2H, SO_2NH_2), 3.86 (s, 2H, ICH_2CO), 3.37 (bs, 1H, CONH); IR: 3308, 3108, 1670, 1310, 1150, 1104, 930 cm^{-1}; MS, m/z 340 (M^+).

3-(Chloroacetamido)benzenesulphonamide (**11**). 3-Nitrobenzenesulphonamide (10.0 g; 0.05 mol) was dissolved in water (350 mL) to which hydrazine hydrate (12.5 g; 0.25 mol) was added. The mixture was refluxed for 45 min and Raney nickel (200 mg) added. Vigorous effervescence was allowed to subside before refluxing the mixture for another 3.5 h. The hot solution was filtered and concentrated to a small volume. A greyish-white precipitate resulted. The product was chilled before being filtered, washed with chilled water and dried *in vacuo* to give 3-aminobenzenesulphonamide, (3-ABS; (6.13 g; 72%), mp 140–142°C. Recrystallisation from hot water gave a purer product, mp 141.5–143.5°C, (lit. 142°C).[91] ^{1}H NMR (DMSO): 7.42–6.79 (m, 4H, 2H of Φ, SO_2NH_2), 6.97–6.64 (m, 2H of Φ), 5.48 (s, 2H, NH_2Φ); IR: 3484, 3388, 3360, 3266, 1620, 1550, 1310, 1152, 1080, 912 cm^{-1}; MS, m/z 172 (M^+).

Chloroacetic anhydride (0.99 g; 5.8 mmol) was added to a solution of 3-ABS (1.0 g; 5.81 mmol) in acetone (45 mL) and refluxed for 2.5 h. The hot solution was filtered, concentrated, and water added (50 mL) to give a white precipitate. Recrystallisation from aqueous ethanol (100 mL; 40:60, v/v) gave sulphonamide **11** (1.08 g, 75%), mp 154–155·2°C (d). ^{1}H NMR (DMSO): 8.18 (bs, 1H, H_5Φ), 7.90–7.45 (m, 3H, H_2, H_4, H_6Φ), 7.37 (s, 2H, SO_2NH_2), 4.28 (s, 2H, $ClCH_2CO$), 3.32 (s, 1H, CONH); IR: 3325, 3286, 3241, 3208, 1683, 1330, 1154, 1090, 920 cm^{-1}. Found: 39.0: H, 3.7; N, 11.5. $C_8H_9ClN_2O_3S$ requires C, 38.6: H, 3.6: N, 11.3%: MS, m/z 248 (M^+).

3-(Iodoacetamido)benzenesulphonamide (**12**). This was made at a yield of 74% from sulphonamide **11** by exchanging the chloride atom for an iodide atom using the same method as that employed in the preparation of **10.** The crude product was recrystallised from aqueous ethanol, mp 199–202°C. ^{1}H NMR (DMSO): 8.14 (bs, 1H, H_5Φ), 7.95–7.46 (m, 3H, H_2, H_4, H_6Φ), 7.33 (s, 2H, SO_2NH_2), 3.92 (s, 2H, ICH_2CO), 3.32 (s, 1H, CONH); IR: 3340, 3312, 3248, 3208, 1670, 1338, 1173, 1096, 909 cm^{-1}. Found: C, 28.4; H, 2.8; N, 8.5. $C_8H_9IN_2O_3S$ requires; C, 28.2; H, 2.6; N, 8.2. MS, m/z 340 (M^+).

4-Iodo-3-(iodoacetamido)benzenesulphonamide (**13**). 3-Amino-4-iodobenzenesulphonamide (0.28 g; 0.94 mmol) in acetone (30 mL) was refluxed with chloroacetic anhydride (0.18 g; 1.05 mmol) for 3 h. The hot solvent was filtered and concentrated down under reduced pressure. On adding water (50 mL), a white precipitate of 4-iodo-3-(chloroacetamido)benzenesulphonamide, (4-ICBS) formed. The product was separated by filtration, washed with chilled water and dried (0.33 g; 94%), mp 212.5–213.5°C. ^{1}H NMR (DMSO): 8.20–7.85 (m, 4H, H_2Φ, H_5Φ, SO_2NH_2), 7.38 (dd, J_{65} = 8.0 Hz, J_{62} = 2.1 Hz, 1H, H_6Φ), 4.34 (s, 2H, $ClCH_2CO$), 3.29 (bs, 1H, CONH); IR: 3346, 3250, 1678, 1334, 1157, 1094, 918 cm^{-1}. Found: C, 25.4; H, 2.1; N, 7.4. $C_8H_8ClIN_2O_3$ requires; C, 25.6; H, 2.1; N, 7.5%. MS, m/z 374 (M^+).

On refluxing 4-ICBS (0.26 g; 0.7 mmol) with sodium iodide (0.13 g; 0.87 mmol) in acetone (30 mL), cooling, and adding water (30 mL), a white precipitate formed. Recrystallisation from aqueous alcohol gave the white fluffy product **13** (0.20 g; 63%), mp 227.2–228.2°C. ^{1}H NMR (DMSO): 8.09 (d, H_{56} = 8.0 Hz, 1H, H_5Φ), 7.82 (d, J_{26} = 2.0 Hz, 1H, H_2Φ); 7.58–7.28 (m, 3H, H_6Φ, SO_2NH_2), 3.91 (s, 2H, ICH_2CO),

3.29 (s, 1H, CONH). D_2O exchange simplified the multiplet to give 1 signal at 7.38 (dd, J_{65} = 8.0 Hz, J_{62} = 2.0 Hz, 1H, $H_6\Phi$). IR: 3359, 3256, 1657, 1347, 1160, 1090, 920 cm^{-1}. Found: C, 20.6; H, 1.9; N, 6.0. $C_8H_8I_2N_2O_3S$ requires; C, 20.6; H, 1.7; N, 6.0%. MS (NH_3 chemical ionization), m/z 466 (M^+).

4-Iodo-3-(acetamido)benzenesulphonamide (**14**). 3-Amino-4-iodobenzenesulphonamide (50 mg; 0.17 mmol) dissolved in dry THF (5 mL), was cooled in ice and acetyl chloride (16 mg; 0.20 mmol) added. The solution was magnetically stirred for 4 h at 2.5°C and left overnight at 10°C. The solvent was evaporated under reduced pressure to a small volume. Warm water was added (40 mL) and the product extracted into chloroform (3 × 50 mL). The chloroform extracts were combined, dried ($MgSO_4$) and completely evaporated, leaving behind a pale white solid. This was dried *in vacuo* to give **14** (43.0mg; 75%), mp 189–193°C. IR: 3374, 3336, 3264, 1662, 1348, 1156, 1092, 925 cm^{-1}. Found: C, 28.8; H, 2.7; N, 8.6. $C_8H_9IN_2O_3S$ requires; C, 28.4; H, 2.7; N, 8.2%. MS, m/z 340 (M^+).

Biological Methods

Isolation and purification of HCA isozymes The two major carbonic anhydrase isozymes, HCA I and HCA II were isolated from time expired human rbc according to a literature method.[99] Briefly, the washed rbc (200 mL) were haemolysed with Tris-Chloride buffer (5 mmol dm^{-3}). The ethanol-chloroform procedure was used to selectively denature the haemoglobin. The crude mixture of the HCA isozymes was concentrated to 20 mL using ultrafiltration (Amicon PM10 membrane) and loaded onto a DEAE-A 50 sephadex anion-exchange column (26 × 260 mm). The upward flowing column was eluted at 4°C with Tris-Chloride buffer (0.05 mol dm^{-3}, pH 8.7) at a flow rate of 10 mL/h (LKB 2132 microperpex pump). The eluent was monitored at an absorbance of 280 nm and collected as 6 mL fractions (LKB, 2070, Ultrorac II fraction collector). Two protein peaks were observed corresponding to the high (HCA II) and low activity (HCA I) isozymes. The fractions from each peak were assayed for specific enzyme activity, combined as appropriate, and concentrated to small volumes by ultrafiltration. These two stock solutions were stored at 4°C and used in the enzyme studies.

Determination of enzyme activity The HCA isozymes were assayed for their esterase activity using *p*-nitrophenyl acetate (*p*-NPA) (1 mmol dm^{-3}). For each assay, an aliquot of *p*-NPA solution (0.1 mol dm^{-3}) in acetone was added to 3 mL of Tris-Sulphate buffer (10 mmol dm^{-3}, pH 8.00, μ = 0.1 mol dm^{-3}) in a 1 cm pathlength quartz cell. An aliquot of one of the stock isozyme solutions (5–10 μL) was added to start the hydrolysis reaction, which was followed at 25.0 ± 0.5°C using a UV spectrometer set at 348 nm. Linear rates were obtained for at least 3–4 min and the non-enzymatic (blank) rate was subtracted to yield the enzyme-dependent rate of *p*-NPA hydrolysis. All assays were performed in triplicate.

Measurement of enzyme concentration During the purification of the HCA isozymes, the protein concentration was determined from the absorbance (A) of the solutions taking $A_{280\,nm}$ (1%) = 16.3 and $A_{280\,nm}$ (1%) = 18.7 for the HCA I and HCA II isozymes, respectively.[40] The concentration of the purified isozymes was determined more accurately by an acetazolamide titration technique.[40] Briefly, an aliquot of the

enzyme solution was assayed for esterase activity as described above, and then in the presence of increasing concentrations of acetazolamide up to that which gave 15–20% inhibition. Linear extrapolation of a plot of acetazolamide concentration (x) versus residual activity (y) yielded the true enzyme concentration as the intercept on the x-axis.

Inhibition studies

Reversible Inhibition The dissociation constant (K_D) of each carbonic anhydrase-sulphonamide complex was determined by measuring the residual activity of the enzyme towards the hydrolysis of *p*-NPA in the presence of increasing amounts of the inhibitor. The assays were carried out in a similar way to those for the acetazolamide titration, in a Tris-Sulphate buffer (10 mol dm^{-3}, pH 8.00, μ = 0.1 mol dm^{-3}), although the spectrophotometric measurements were done at 400 nm using 1 cm path-length plastic curvettes. A constant volume of a stock enzyme solution was used in the assay of any given inhibitor. The apparent K_D values were obtained from a Dixon-plot[100] or an α-plot.[101] In either case, the best fit was computed by a least squares regression procedure. Generally, Dixon-plots were suitable for low affinity sulphonamides, whereas α-plots were appropriate for high affinity sulphonamides; in the latter case the initial inhibitor concentration is not assumed to be the same as that free in solution.

Irreversible Inhibition Further experiments were carried out with potential irreversible inhibitors and their unreactive analogues. Molar equivalents of the HCA isozymes (100–200 μL of stock solution) and of the sulphonamides (**6–14,** Table VII) (1–3 μL in acetone) were incubated at 25.0 $\pm$ 0.5°C in Tris-Sulphate buffer (10 mmol dm^{-3}, pH = 8.0, μ = 0.1 mol dm^{-3}). Control mixtures without inhibitor, were incubated in parallel. All mixtures were incubated for 96 h. Aliquots (2–5 μL) were withdrawn at various time intervals up to 96 h and assayed for esterase activity.

RESULTS AND DISCUSSION

Pure sulphonamides were generally obtained in good yields. The apparent inhibitor dissociation constant (K'_D) determined for each sulphonamide with the two carbonic anhydrase isozymes acting as esterases, are presented in Table VII. While HCA may not act as an esterase *in vivo*, the enzyme does catalyse the hydrolysis of *p*-NPA to provide a very convenient *in vitro* assay. Inhibition of esterase activity parallells the inhibition of CO_2 hydration. The ionisation constant (pK_a) and partition coefficient (Log *P*) values are also shown in Table VII for some of these sulphonamides. The K'_D values determined for acetazolamide (**15**) are in close agreement to those reported in the literature.[55,57,101]

Reversible Binding

The simplest aromatic sulphonamide synthesised, *p*-iodobenzenesulphonamide, pIBS(**1**) was found to be amongst the most potent of the inhibitors. Unlike any of the other sulphonamides, pIBS binds to the HCA I and HCA II isozymes with almost equal avidity. Binding of pIBS to the HCA II isozyme is some ten times stronger than *p*-chlorobenzenesulphonamide (K'_D = 1.36 × 10^{-8} mol dm^{-3} versus 1.21 × 10^{-7}

mol dm^{-3}) and one hundred times stronger[57] than benzenesulphonamide [K'_D = 1.54 × 10^{-6} mol dm^{-3}]. The high affinity for the HCA isozymes cannot be attributed solely to the slight increase in acidity of the sulphonamide group (pK_a = 10.06) as compared to that in benzenesulphonamide [pK_a = 10.2].[101] It seems most probable that the para-substituted aryl iodide atom in pIBS interacts favourably with active-site amino acid residues.

Sulphonamides **2** and **3** incorporate a second aromatic ring and were designed to provide enhanced enzyme affinity. In fact this was only observed for the interaction between **2** and HCA II. An interesting feature of **2** is the ten-fold difference in inhibiton for the two isozymes. This inhibition distinction is not observed for **3** but is seen again for **5** and **16,** and for **13, 14** and **15,** i.e. in cases where either a sulphonyl group is directly attached to the aromatic or heterocyclic ring opposite the primary sulphonamido group, or an amide is substituted in the meta position on the aromatic ring or opposite the sulphonamide on the heterocyclic ring. *p*-Acetamidobenzenesulphonamide also exhibits differential inhibition but the haloacetamido analogues (**7–10**) do not. Sulphonamides **1–5** and **16** are candidates for reversibly binding carriers. Consideration of the K'_D values and the partition coefficients (Table VII) would favour the choice of **1, 4** and **16.** The lipophilicity of these three sulphonamides should ensure rapid red cell uptake while the tight binding to carbonic anhydrase (K'_D = 10^{-8}–10^{-10} mol dm^{-3}) satisfies a further prerequisite. Further *in vitro* and *in vivo* studies for sulphonamide **1** as a specific radioisotopic carrier for use in red cell labelling have been reported elsewhere.[102,103]

Irreversible Binding

Sulphonamides **6–14** were prepared to investigate the irreversible inhibition of the carbonic anhydrase isozymes. However, in initial rate studies (the first 4 min), reversible binding was observed for all of these inhibitors as indicated by their K'_D values (Table VII). In its initial reversible binding, *p*-acetamidobenzenesulphonamide **(6)** is considerably less potent than **1**. However, the replacement of a methyl proton in **6** by a halogen atom results in a much more powerful inhibitor **(7, 9, 10,** Table VII); even more encouraging is the retention of this enhanced potency when an aromatic iodide **(8)** is included, for this atom may be exchanged for a radiolabel. Placing the haloacetamido group meta to the sulphonamido group gives weaker inhibitors (compare **11** with **7,** and **12** with **10,** respectively), although inclusion of an aromatic iodide atom para to the sulphonamido group, as in **13** serves to restore the potency somewhat. However, **13** is still a hundred times less potent than **1** at inhibiting the esterase activity of HCA I, suggesting that steric repulsion is probably encountered in the active-site by the meta-iodoacetamido moiety. The adverse effect of changing from para- to meta-substitution has been reported previously.[84]

The irreversible inactivation of carbonic anhydrase was monitored by individually preincubating sulphonamides **7–13** with stoichiometric amounts of the two HCA isozymes; sulphonamides **6** and **14** were used as controls. The enzyme activity remaining in the incubation mixtures was determied by subsequent dilution in a standard assay. Results are presented in Tables VIII and IX for preincubation periods up to 96 h. Over this period, para-substituted sulphonamides **7–10** show no irreversible inhibition of either isozyme. This is consistent with the result reported in the literature for a para-substituted sulphonamide such as *p*-chloroacetamidobenzenesulphonamide which also failed to irreversibly inactivate the HCA I isozyme, although it

TABLE VIII

Changes in HCA I ester hydrolysis activity at pH 8.00, 25°C upon preincubation with stoichiometric concentrations of some sulphonamide inhibitors

Sulphonamide No.	Theoretical % EA remaining in: IM	ASSAY	HCA I Isozyme % EA remaining at: 0 h	24 h	48 h	96 h
6.	6.4	84.1	79.2 ± 3.9	74.9 ± 3.7	-	73.3 ± 3.7
7.	2.0	47.5	43.8 ± 2.2	44.5 ± 2.2	-	39.2 ± 2.0
8.	1.9	44.5	41.1 ± 2.0	38.5 ± 1.9	-	34.1 ± 1.7
9.	2.0	46.5	38.4 ± 1.9	38.5 ± 1.9	-	35.6 ± 1.8
10.	1.2	31.3	23.5 ± 1.2	25.6 ± 1.3	-	29.4 ± 1.5
11.	10.8	95.4	85.5 ± 4.3	16.8 ± 0.5	5.7 ± 0.3	1.2 ± 0.1
12.	9.3	93.9	79.9 ± 4.0	2.3 ± 0.1	0.0 ± 0.01	0.0 ± 0.01
13.	7.2	90.3	87.2 ± 4.4	1.3 ± 0.1	0.9 ± 0.05	0.0 ± 0.01
14.	4.0	59.1	69.0 ± 4.1	73.5 ± 2.2	76.7 ± 3.8	74.3 ± 3.0

The incubation mixture (IM) was prepared by adding a molar equivalent of the sulphonamide in acetone (1–3 μL) to a sample (100–130 μL) of the stock HCA I isozyme solution (271 μmol dm^{-3}). The theoretical percentage enzyme activities (% EA) remaining in IM and after dilution in the assay are those values calculated for reversible inhibition, using the measured dissociation constants given in Table VII.

did bind reversibly[60] with $K'_D = 2.5 \times 10^{-6}$ mol dm^{-3} at pH 7.6. Lack of any active-site-directed, irreversible inhibiton by these para-substituted benzenesulphonamides suggests that when the sulphonamido group is reversibly bound to the active-site of the isozymes, the reactive haloacetamido group is in an unfavourable geometric orientation. This precludes any alkylation of the active-site histidine residues. The apparent time-dependent increase in activity of HCA II observed with the control **14** is anomalous (Table IX).

The meta-haloacetamido substituted analogues **11–13** rapidly inactivate the HCA I isozyme (Figure 2 and Table VIII) in an irreversible, time-dependent manner with half-lives ($t_{1/2}$) of 8.3 ± 2.0 h for **11**, 5.9 ± 1.5 h for **12**, and 1.9 ± 0.2 h for **13**. The differences in the rates of reaction for **11** and **13** simply relate to the fact that the aryl iodide atom in **13** is a much better leaving group than the aryl chloride atom in **11**. However, the irreversible reaction with HCA II (Figure 3 and Table IX) is much

TABLE IX

Changes in HCA II ester hydrolysis activity at pH 8.00, 25°C upon preincubation with stoichiometric concentrations of some sulphonamide inhibitors

Sulphonamide No.	Theoretical % EA remaining in: IM	ASSAY	HCA II Isozyme % EA remaining at: 0 h	24 h	48 h	96 h
6.	8.4	86.9	84.8 ± 4.2	91.7 ± 2.7	-	85.6 ± 4.3
7.	5.9	81.8	77.6 ± 1.3	72.7 ± 5.0	-	83.2 ± 2.5
8.	6.4	84.0	81.8 ± 1.2	79.9 ± 1.0	-	85.1 ± 2.3
9.	5.7	81.8	72.3 ± 3.6	70.6 ± 3.5	-	75.0 ± 1.5
10.	2.6	56.2	42.5 ± 2.1	47.3 ± 2.4	-	64.8 ± 1.0
13.	5.1	69.6	77.8 ± 3.9	64.9 ± 3.2	60.3 ± 0.1	55.2 ± 2.8
14.	5.0	80.0	85.0 ± 3.9	89.1 ± 4.4	92.3 ± 4.0	98.5 ± 4.9

An aliquot (100 μL) of stock HCA II isozyme (26 μmol dm^{-3}) and a molar equivalent of the sulphonamide solution (1–3 μL) were used for making the incubation mixture (IM). Theoretical percentage enzyme activities (% EA) remaining in IM and after dilution in the assay represent values expected for reversible inhibition.

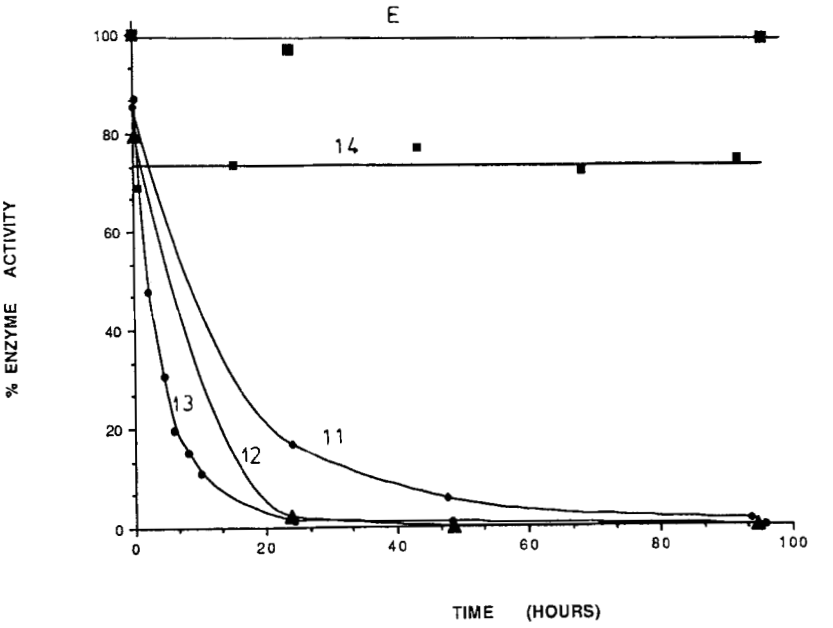


FIGURE 2 Effect of preincubation with sulphonamide inhibitors **11, 12, 13** and **14** on the carbonic anhydrase isozyme, HCA I hydrolysis of *p*-nitrophenyl acetate. Assays were conducted at 25°C as described in the Experimental Section. **E** denotes preincubations with the isozyme but no inhibitor.

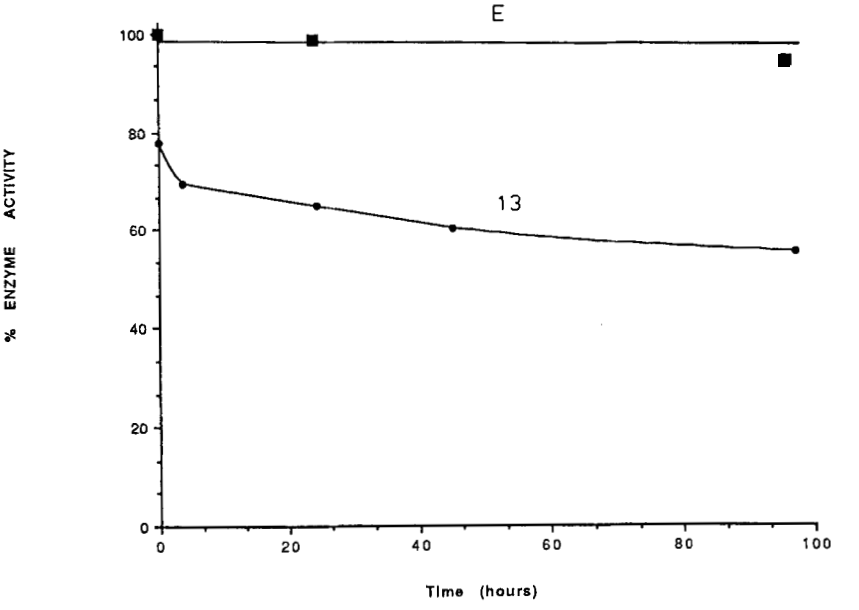


FIGURE 3 Effect of preincubation with sulphonamide inhibitors **13** on the carbonic anhydrase isozyme, HCA II hydrolysis of *p*-nitrophenyl acetate. Assays were conducted at 25°C as described in the Experimental Section. **E** denotes preincubations with the isozyme but no inhibitor.

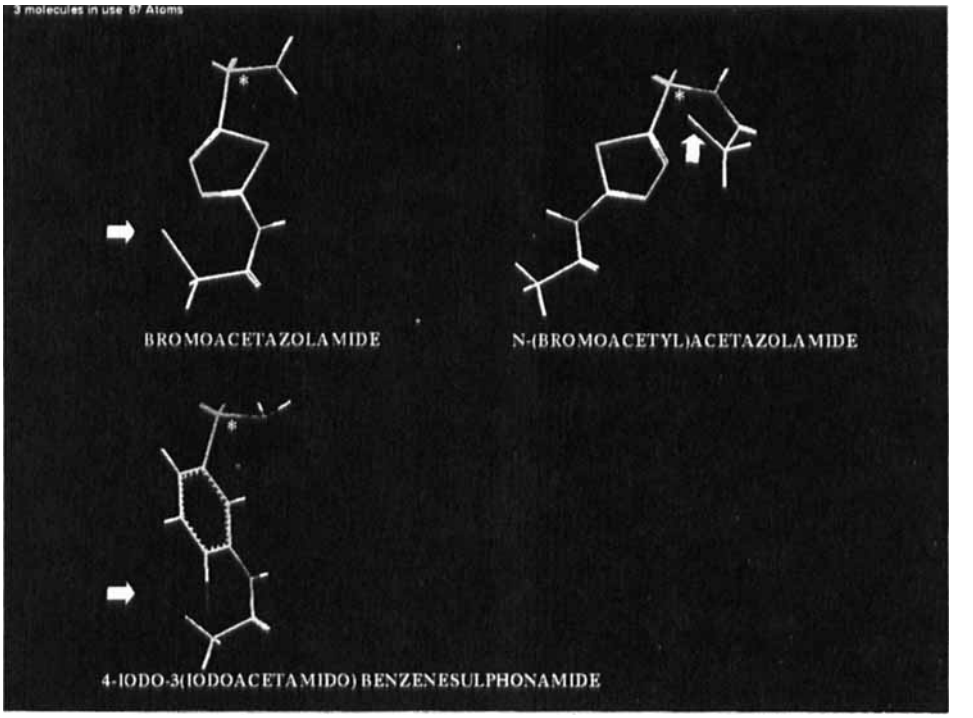


FIGURE 4 The IRIS Workstation Silicon Graphics Computer drawn structures of the three carbonic anhydrase sulphonamide inhibitors, Bromoacetazolamide, *N*-(Bromoacetyl)acetazolamide and 4-Iodo-3-(iodoacetamido)benzenesulphonamide **(13)**. The astrisk (*) indicates the position of the sulphonamido group and the arrow indicates the position of the halogen atom which would be eventaully displaced in the irreversible inactivation of the target enzyme. (See colour plate at back of issue).

slower ($t_{1/2}$**(13)** $\pm$ 180 h) presumably reflecting geometric differences of the two isozymes with respect to the active-site residues.

Sulphonamides such as bromoacetazolamide slowly alkylate the 3′ N-Histidine 64 residue in HCA I and rapidly alkylate the same histidine residue in HCA II.[56,62] On the other hand, *N*-(bromoacetyl)acetazolamide rapidly alkylates the 1′ N-Histidine 67 residue in HCA I but does not modify any residue in HCA II.[56] 3-Dimensional structures of bromoacetazolamide, 4-iodo-3-(iodoacetamido)benzenesulphonamide**(13)**, and *N*-(bromoacetyl)acetazolamide (using IRIS Workstation Silicon Graphics computer, run on "Quanta" supplied by Polygen) show that the former two CA inhibitors are very similar at least in the relative position of the sulphonamido group with respect to the halogen atom that would subsequently be displaced in the alkylation reaction (Figure 4). So, Histidine 64 in HCA I is the most probable residue to which the sulphonamide inhibitors **11–13** are covalently linked; alkylation being particularly rapid for **13** which is in contrast to the slow reaction time for bromoacetazolamide.

In this study, sulphonamide **13** is the only candidate suitable for use as an irreversibly bound carrier. However, prior to enzyme alkylation, it is only a moderate reversible inhibitor (K'_D: HCA I = 1.5×10^{-6} mol dm^{-3}; HCA II = 0.70×10^{-7} mol dm^{-3} at pH 8.00, 25°C) which could vitiate its *in vivo* use.

CONCLUSIONS

On a theoretical basis, the enzyme-inhibitor approach would seem to be ideal for red cell labelling. It should be possible to capitalise on the comprehensive appreciation of the carbonic anhydrase - sulphonamide interaction to design improved, specific short, intermediate and long-term carriers. The two appropriate reversible inhibitors synthesised and evaluated *in vitro* were *p*-iodobenzenesulphonamide**(1)** and 4-[(4-iodophenyl)thio]benzenesulphonamide**(4)**. The iodo analogue of 5-[(4-bromophenyl)-sulphonyl]thiophene-2-sulphonamide**(16)** should be equally potent and therefore also useful. Although another sulphonamide, 4-iodo-3-(iodoacetamido)benzenesulphonamide**(13)** was a weaker carbonic anhydrase inhibitor, it did cause a relatively fast irreversible inactivation of the HCA I isozyme, thus showing potential for use in longer term studies.

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