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CALMODULIN ANTAGONISTS OF IMPROVED POTENCY AND SPECIFICITY FOR USE IN THE STUDY OF CALMODULIN BIOCHEMISTRY

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Abstract—Syntheses are described for a range of *N*-(ω-aminoalkyl)-5-iodo- and -5-cyanonaphthalene-1-sulphonamides. The selective activity of these compounds as inhibitors for calmodulin-dependent phosphodiesterase (EC 3.1.4.17) is compared with their activity for the calmodulin-independent but calcium-dependent enzymes protein kinase C and transglutaminase (EC 2.3.2.13). The results show a drastic improvement in the selectivity of effect for the 5-iodo-compounds compared with the widely-used drug, W7, *N*-(6-aminohexyl)-5-chloronaphthalene-1-sulphonamide.

Calmodulin mediates many of the diverse intracellular actions of calcium and roles for it have been suggested in cyclic nucleotide metabolism, hormone secretion, cell motility and contractility and cell division [1]. A range of chemically dissimilar drugs [2] will inhibit calmodulin activity and as a consequence have been employed as probes in the study of calmodulin-dependent processes in cells. However, many such drugs, for example the phenothiazines commonly used for this purpose [2], the substituted naphthalenesulphonamides, such as W7 [3], and others, such as R24571, "calmidazolium" which are particularly potent [4], will inhibit other non-calmodulin-dependent processes at concentrations only slightly higher than those required for calmodulin inhibition. In view of this, other important calcium-dependent but calmodulin-independent enzyme activities must also be considered in the current picture of intracellular regulation when using such probes. In particular, the calmodulin-independent but calcium-dependent enzyme protein kinase C, which is thought to be fundamental to many intracellular processes [5], is inhibited by a number of calmodulin antagonists over the concentration range used in studies involving intact cells [4]. For example, the most potent of the commonly available substituted naphthalenesulphonamides *N*-(4-aminohexyl)-5-chloronaphthalene-1-sulphonamide (W7) has been reported to have only a factor of five separating the potency of the drug against calmodulin-dependent phosphodiesterase activity from that against protein kinase C [6]. Similarly, the calcium-activated enzyme transglutaminase thought to be important in cellular processes, such as receptor mediated endocytosis [7] and stimulus secretion coupling [8, 9], is also reported to be inhibited by W7 [10] at concentrations similar to those required to bring about calmodulin antagonism.

Accordingly, the aim of this study was to develop calmodulin antagonists of improved specificity for use as pharmacological tools in the study of calmodulin biochemistry and also for evaluation as potential carcinostatic/antiproliferative agents. The approach we took was to extend the well-documented structure activity studies of Hidaka *et al.* [3] based on substituted naphthalenesulphonamides.

We report here that improvement in the potency of such drugs as calmodulin antagonists can be achieved with a very significant improvement in specificity.

MATERIALS AND METHODS

Materials

5-Aminonaphthalene-1-sulphonic acid, α,ω-diamines and *N*-dansylcadaverine were purchased from Fluka A.G. Chemische Fabrik and other chemicals from Aldrich Chemical Co. Ltd. Cyclic [^{3}H] AMP, [α^{32}P] ATPand [1,4-^{14}C]Putrescine were purchased from Amersham International plc. Calmodulin-dependent beef heart phosphodiesterase was obtained from Boehringer Mannheim Co. All other biochemicals were obtained from Sigma Chemical Co.

Methods

Confirmation of chemical structure. The purity and structure of each of the synthesised compounds was confirmed by determination of melting points and spectroscopic analysis using NMR and mass spectroscopy.

Proton NMR spectra were recorded using a Perkin-Elmer R34 instrument at 220 MHz with TMS as internal reference. ^{19}F NMR spectra were recorded at 75.2 MHz on a Bruker WP80 SY machine with $CFCl_3$ as external reference. Low resolution mass spectra were obtained with a Kratos MS25 machine and infrared spectra were recorded by Nujol mulls on a Perkin-Elmer 157G or a 457G instrument. Melting points were determined with a Kofler hot stage microscope and are otherwise uncorrected.

Synthesis of compounds

2,4,6-Isopropylbenzenesulphonamide was prepared as previously described [11].

5-Cyanonaphthalene-1-sulphonyl chloride was prepared from 5-amino-naphthalene-1-sulphonic acid as previously described [11], sodium 5-cyano-naphthalene-1-sulphonate being converted into the chloride by treatment with phosphoryl chloride to give brownish crystals from glacial acetic acid m.p. 144.5° (lit. [11] m.p. 140.3°).

5-Chloronaphthalene-1-sulphonyl chloride was prepared as previously described [12, 13] as dark brown crystals from glacial acetic acid, m.p. 940 (lit. [14] m.p. 95°).

5-Iodonaphthalene-1-sulphonyl chloride was prepared from 5-iodonaphthalene-1-sulphonic acid by reacting its anhydrous sodium salt with phosphoryl chloride at 80°. The product was crystallised from glacial acetic acid as yellow crystals, m.p. 115° (lit. [15] 114°. (Found: C, 34.1; H, 1.75; Cl, 9.65; I, 35.05; S, 9.8. Calc. for $C_{10}H_6ClIO_2S$: C, 34.07; H, 1.72; Cl, 10.06; I, 36.00; S, 9.10%).

5-Iodonaphthalene-2-sulphonyl chloride was prepared from sodium 5-iodonaphthalene-2-sulphonate as above, m.p. 97.9° (lit. [15] m.p. 102°).

1-Trifluoromethylnaphthalene was obtained from 1-iodonaphthalene by treatment with copper and iodotrifluoromethane in pyridine [16] as a pale yellow oil bp_4 70.2°. (Found: C, 67.3; H, 3.55. Calc. for $C_{11}H_7F_3$: C, 67.35; H, 3.60%).

5-Trifluoromethylnaphthalene-1-sulphonyl chloride was prepared by chlorosulphonylation [17] of 1-trifluoromethylnaphthalene [16] and the crude product crystallised from glacial acetic acid as cream coloured prisms m.p. 192.4°. (Found: C, 44.5; H, 2.55; Cl, 12.05; S, 11.05. Calc. for $C_{11}H_6ClF_3O_2S$: C, 44.97; H, 2.06; Cl, 11.76; S, 10.91%).

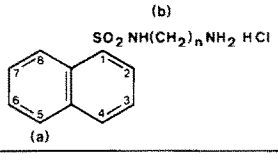
3-Trifluoromethylbenzenesulphonyl chloride was prepared [18] by the chlorosulphonylation of benzotrifluoride to give an oil, bp_{12} 103.8°. The product was further characterised by treatment with liquid ammonia to give 3-trifluoromethylbenzenesulphonamide m.p. 106.8° (lit. [19] m.p. 111.3°. (Found: C, 37.75; H, 2.8; N, 6.05; S, 14.45. Calc. for $C_7H_6F_3NO_2S$: C, 37.34; H, 2.69; N, 6.22; S, 14.24%).

General preparation of N-(ω-aminoalkyl)-arenesulphonamides. The ω-diaminoalkane (60 mmol) was dried by azeotropic distillation with benzene and dissolved in dry dioxane (80 ml). A solution of the arenesulphonyl chloride (10 mmol) in dry dioxane (40 ml) was added with vigorous stirring during 30 min at 10° then, after 1 hr, the solution was warmed to 80° for 30 min, cooled, and evaporated *in vacuo*. After removal of excess amine and salts by trituration with water, the solid residue was extracted with hot methanol (200 ml) and the solution acidified to pH 2 with HCl and evaporated to give the crude product which was crystallised from ethanol or aqueous methanol [20].

N-(t-butyl)-5-iodonaphthalene-1-sulphonamide. A solution of 5-iodonaphthalene-1-sulphonyl chloride (6.6 g, 20 mmol) in dichloromethane (80 ml) was added dropwise to a stirred solution of *t*-butylamine (2.72 g, 40 mmol) in the same solvent (50 ml) at 0°. After 48 hr the solution was evaporated *in vacuo* and the residue extracted with ether. The extract was washed with dilute HCl, water, dried and evaporated. The product was crystallised from toluene:hexane (1:1, v/v) as yellow needles (5.0 g, 68%) m.p. 238–241°. (Found: C, 42.9; H, 4.3; I, 32.4; N, 3.6; S, 8.2. $C_{14}H_{16}INO_2S$ requires C, 43.17; H, 4.44; I, 32.64; N, 3.60; S, 8.22%).

Calcium/calmodulin-dependent phosphodiesterase

Table 1. The effect of modifying (a) the choice of substituent at C_5 and (b) the composition and position of the sulphonamide side chain on calmodulin antagonist activity.



Compound	Name	(a)	(b)	IC_{50} $\bar{x} \pm$ SEM (N) (μM)
a	5-chloro-1-C_6 (W7)	Cl	$SO_2NH(CH_2)_6NH_2$	29 + 4(4)
b	5-iodo-1-C_4	I	$SO_2NH(CH_2)_4NH_2$	8 + 2(3)
c	5-iodo-1-C_6	I	$SO_2NH(CH_2)_6NH_2$	29 + 6(6)
d	5-iodo-1-C_7	I	$SO_2NH(CH_2)_7NH_2$	7 + 0.6(3)
e	5-iodo-1-C_8	I	$SO_2NH(CH_2)_8NH_2$	3 + 0.6(4)
f	5-iodo-2-C_8	I	$SO_2NH(CH_2)_8NH_2$	4 + 1.4(5)
g	5-iodo-1-C_9	I	$SO_2NH(CH_2)_9NH_2$	3 + 0.4(3)
h	5-iodo-1-C_{10}	I	$SO_2NH(CH_2)_{10}NH_2$	4 + 0.9(3)
i	5-iodo-1-C_{12}	I	$SO_2NH(CH_2)_{12}NH_2$	0.7 + 0.1(3)
j	5-CF_3-1-C_6	CF_3	$SO_2NH(CH_2)_6NH_2$	100 + 10(3)
k	5-cyano-1-C_6	CN	$SO_2NH(CH_2)_6NH_2$	59 + 10(5)
l	5-cyano-1-C_8	CN	$SO_2NH(CH_2)_8NH_2$	2 + 0.1(3)
m	5-cyano-1-C_9	CN	$SO_2NH(CH_2)_9NH_2$	3 + 0.7(4)
n	5-cyano-1-C_{10}	CN	$SO_2NH(CH_2)_{10}NH_2$	5 + 1.6(5)
o	5-iodo-1^+	I	$SO_2NHC(CH_3)_3$	20 + 2(3)
p	3-CF_3-1-C_6^{++}	CF_3	$SO_2NH(CH_2)_6NH_2$	250 + 62(3)

Compounds o and p differ from a–n above in that o^+ is a *t*-butyl derivative of b, p^{++} is 3-trifluoromethyl-benzene-sulphonamide, prepared as a molecule having a similar spatial relationship between the halogen and the sulphonamide group as that in W7 (see Table 2 for structure).

assay. The calmodulin inhibitory potency of the compounds prepared (Table 1) was determined with commercially-available (Boehringer-Mannheim) beef heart cyclic nucleotide phosphodiesterase (EC 3.1.4.17) using the methodology previously described [21]. Assays were performed in triplicate. Each drug was examined in not less than three separate assays.

Calcium-dependent transglutaminase assay. Particle free supernatant from extracts of rat liver [18] was used as a source of transglutaminase. Transglutaminase activity was measured using the procedure previously described [8]. Assays were performed in triplicate.

Calcium-dependent protein kinase C assay. The potency of compounds as inhibitors of protein kinase C was determined by measuring protein kinase C activity in extracts prepared from rat spleen. Rat spleen was homogenised in 3 vol. of Tris/HCl (20 mM, pH 7.4), EGTA (2 mM), 2-mercaptoethanol (50 mM) with a Potter-Elvehjem homogeniser. The homogenate was centrifuged at 71,000 *g* for 45 min at 4° and the supernatant retained. This preparation was diluted 1 : 10 with Tris/HCl (20 mM, pH 7.4) for use in the protein kinase C assays. The standard reaction mixture (100 μl) contained the following components: Tris/HCl (20 mM, pH 7.4), Mg $(OAc)_2$ (5 mM), histone H1 (200 μg ml^{-1}). [γ-^{32}P] ATP (10 μM, 0.6 Ci mmol^{-1}), $CaCl_2$ (98 μM), EGTA (48 μM), 2-mercaptoethanol (1.2 mM), phosphatidylserine (16 μg ml^{-1}), 1,2-diolein (2.4 μg ml^{-1}), and the appropriate concentration of test compound. Reactions were started by the addition of spleen extract (24 μl, approx 25 μg protein). The incorporation of ^{32}P from [γ-^{32}P] ATP into histone H1 was measured by the modified procedure of Lord and Ashcroft [22] whereby phosphorylated proteins were recovered by precipitation with trichloroacetic acid (5% w/v) containing osmium tungstate (0.25% w/v). Protein kinase C activity was calculated by subtracting the phosphorylation obtained in the presence of Ca^{2+} alone from that obtained in the presence of Ca^{2+}, 1,2-diolein, and phosphatidylserine over a period of 2 min incubation at 37°. Addition of phospholipid and diolein stimulated enzyme activity by approx 60%. Under these conditions, the incorporation of ^{32}P was linear with time, and the standard inhibitor of protein kinase C, trifluoperazine [6] was found to give 50% inhibition of enzyme activity at a concentration of 74 μM. Assays were performed in triplicate.

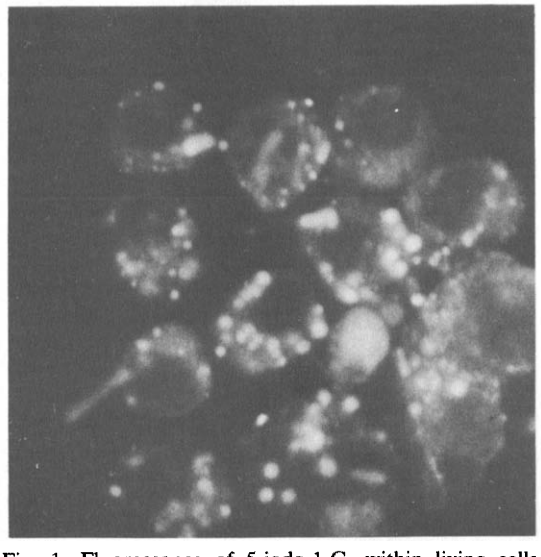


Fig. 1. Fluorescence of 5-iodo-1-C_8 within living cells. Human leukaemic (K562) lymphocytes were exposed to 100 μM 5-iodo-1-C_8 in phosphate-buffered saline. The picture, taken at 20 min, shows the maximum fluorescence obtained which developed over 15–20 min.

Addition of test compounds to enzyme assays. Many of the naphthalenesulphonamides were poorly water soluble and dimethylsulphoxide, DMSO, was used to dissolve these drugs. Appropriate dilutions of DMSO (up to 1% final) were added to all assays where such drugs were present. That DMSO itself did not affect the calmodulin-inhibitory potency of the drugs was determined from a comparison of some of the more soluble drugs which were assayed in the presence and in the absence of DMSO.

Fluorescence microscopy. Compounds were examined for fluorescence inside cultured cells (human K562 leukaemic lymphocytes) using an Orthomat microscope camera on an Orthoplan microscope (Ernst Leitz GMBA Wetzlar, F.R.G.). Cells were washed free of medium, resuspended in phosphate-buffered saline and compounds added to this suspension. A UV filter system and Fujichrome 400 ASA film were used to record this fluorescence as illustrated in Fig. 1.

RESULTS AND DISCUSSION

Physical nature of compounds synthesized

Fifteen naphthalenesulphonamides were prepared (see Table 1) in addition to W7. They were designed to explore three structural variations on *N*-(6-aminohexyl-5-chloronaphthalene-1-sulphonamide, W7. First, the choice of substituent at C-5, restricted by Hidaka to the 5-chloro- and 5-bromo-compounds, was expanded to include 5-iodo, 5-cyano, and 5-trifluoromethyl groups. Secondly, the length of the side chain was varied from four to twelve carbon atoms. Thirdly, the sulphonamide function was moved from the 1- to the 2-position of naphthalene to allow evaluation of any isomeric advantage. The 3-trifluoromethylbenzenesulphonamide was prepared as a molecule having a similar spatial relationship between the halogen function and the sulphonamide group as that in W7 while the *t*-butyl derivative was prepared for evaluation as a possibly inactive control compound.

The water solubility of the drugs was found to decrease with increasing length of the side chain (for the iodo- and chloro- series of compounds) and also to decrease in the series 5-chloro > 5-iodo > 5-cyanonaphthalenesulphonamides.

Experience with the crystallisation of the drug hydrochlorides indicated that there is a general lowering of alcohol solubility for the odd-numbered side chains compared to their even-numbered neighbours. The diminished water solubility of the longer chain compounds was circumvented for enzyme assay purposes by the use of DMSO (up to 1% final in assay) as co-solvent but this problem may possibly limit their potential as drugs for use *in vivo*.

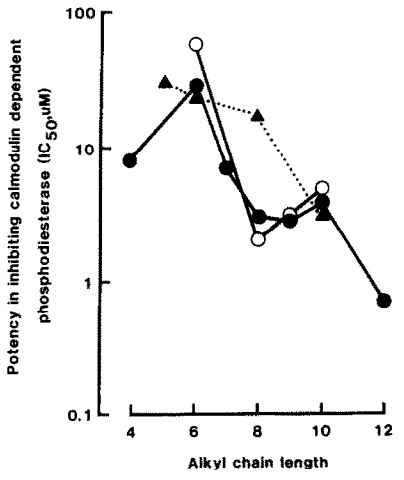


Fig. 2. The variation in potency of compounds as inhibitors of calmodulin-dependent phosphodiesterase as a function of the length of the alkyl chain: ●, 5-iodo-compounds; ○, 5-cyano-compounds; and – – ▲ – –, 5-chloro-compounds. Data for 5-chloro compounds were taken from Hidaka *et al.* [3].

Finally, it should be mentioned that all of the 5-iodonaphthalene-1-sulphonamides proved to have a marked fluorescence. This was found to show maximum emission at 345 nm and maximum excitation at 293 nm in water and is visible to the naked eye as a yellow fluorescence in daylight. This emission is, if anything, enhanced when such compounds enter cells (Fig. 1). Entry of 5-iodo-1-C_8 into cells (which had barely detectable autofluorescence) occurred within a few minutes as observed under a fluorescence microscope. Maximum fluorescence was obtained within 15–20 min and was at least 10 times greater than that attributable to the present compound W7. Figure 1 shows the fluorescence obtained after exposure of K562 human leukaemic lymphocytes to 100 μM 5-iodo-1-C_8 for 20 min. The non-fluorescent intracellular areas correspond to the position and size of the nuclei for these cells. The densely staining structures which appear to be located at or near the surface membrane have not yet been identified. This degree of fluorescence may prove to be useful for the future analysis of the location of calmodulin–drug interactions within living cells.

Ability of naphthalenesulphonamides to inhibit calmodulin. The calmodulin antagonist potency of these compounds was determined by their ability to inhibit calmodulin-dependent beef heart phosphodiesterase activity, which is one of the best established calmodulin-dependent enzyme processes [2]. The results are shown in Table 1. From these data, it is evident that the 5-iodo- and 5-cyano-substituents (c, k) can effectively replace the chlorine atom at C-5 in W7 (a) but that the trifluoromethyl group (j) is rather less active. There is no apparent inprovement in potency for the 5-iodonaphthalene-2-sulphonamide (f) relative to the 1,5-isomer (e)—a finding that contrasts with a report to the contrary for the 5-chloro-series of drugs [3]. Most significantly, the variation in calmodulin inhibition with variation in chain length (Fig. 2) (compounds b–i and k–n) is not as simple as that reported by Hidaka [3] for a limited number of compounds.

In particular, there seems to be a minimum activity for the C-6 chain and a sudden, fivefold increase in activity in going from C-10 to C-12 (h–i) which deserves to be further explored. However, this gain in activity with increasing chain length for the 5-iodo-series has to be offset against the diminished water

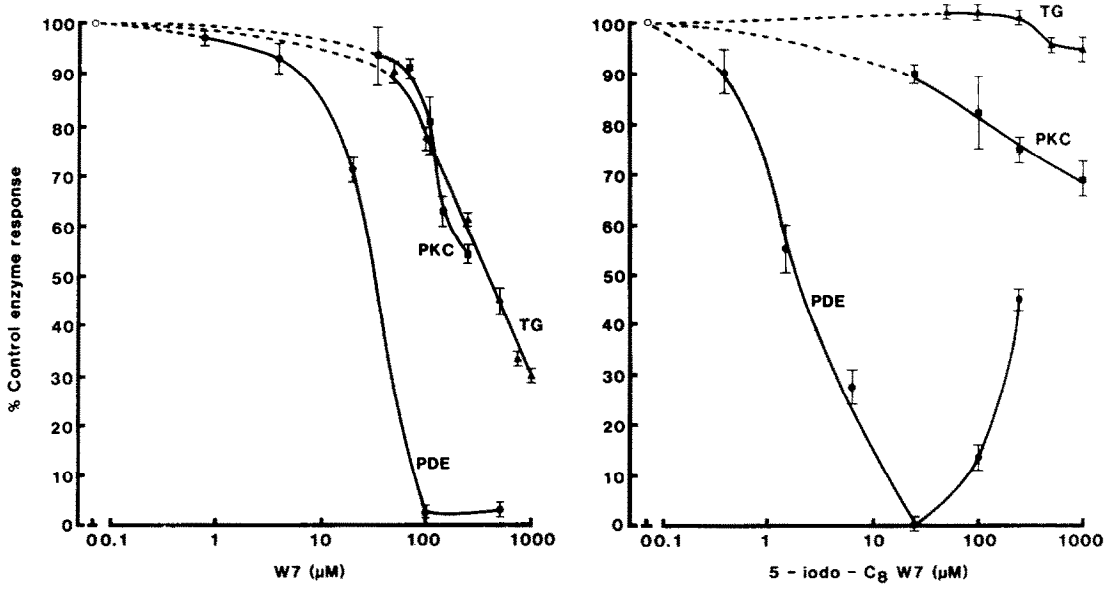
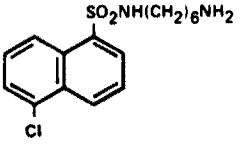
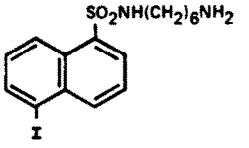
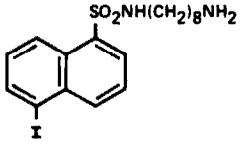
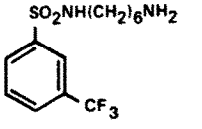
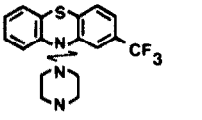
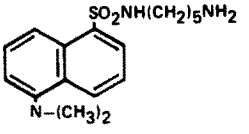
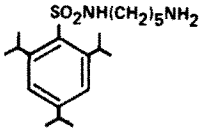
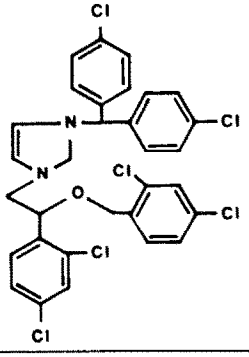


Fig. 3. A comparison of the specificity of action of W7 and 5-iodo-1-C_8 shown by their ability to inhibit calcium, calmodulin-dependent phosphodiesterase (PDE), calcium-dependent, calmodulin-independent rat liver transglutaminase (TG) and calcium-dependent, calmodulin-independent rat spleen protein kinase C (PKC). Results shown are means ± SEM of triplicate determinations of representative experiments. Results based on three such experiments are shown in Table 2.

Table 2. Inhibition of calmodulin dependent and independent enzymes by drugs

Name	Structure	IC_{50} (μm) (PDE)	(PKC)	(TG)
W7 (a)		29 ± 2	342 ± 47 [138 in (6)]	362 ± 93
5-iodo-1-C_6 (c)		29 ± 6	⩾1000	506 ± 59
5-iodo-1-C_8 (e)		3 ± 0.6	>1000	>1000
3-CF_3-1-C_6 (p)		250 ± 62	>1000	>500
Trifluoperazine		4 ± 2	74 ± 7	>1000
N-Dansylcadaverine		387 ± 9	⩾1000	43 ± 7
N-TIPS cadaverine		26 ± 15	200 (n = 2)	20 ± 2
Calmidazolium		0.03 ± 0.02 [0.1 in (4)]	[5.3 in (4)]	—

Phosphodiesterase (PDE), protein kinase C (PKC) and transglutaminase (TG) were assayed as described in Materials and Methods. Results shown are means of a minimum of 2, usually 3, experiments with each drug. Values shown in square brackets represent those obtained from the literature. *N*-TIPS is 2, 4, 6,-tri-isopropylbenzene sulphonamide. Values shown are means ± SEM based on 3 separate experiments for all except one case where N = 2 as indicated.

solubility for these compounds. It is important to discover whether this enhancement of activity stems from an improved hydrophobic interaction of the drug with calmodulin or from greater flexibility of the side chain.

One curious phenomenon was manifest by all of the iodo-compounds. While low concentrations ($\leq 50\ \mu M$) of these drugs (b–i) inhibit the calmodulin-dependent phosphodiesterase activity down to basal (i.e. calmodulin-independent) activity, at higher concentrations of the drugs ($\geq 50\ \mu M$) there is a restoration of enzyme activity approaching 50% of the maximum activity observed in the absence of drug (see Fig. 3). This effect appears (i) to be unrelated to the potency of the drug (b–i), (ii) to be unique in the whole range of compounds studied to the iodo-series, (iii) not to be an artefact of scintillation counting since the effect was not manifest in the presence of boiled, inactive phosphodiesterase—thereby excluding the possibility that fluorescence of the drugs affected the final scintillation counting in the assay, and (iv) did not occur in the absence of calmodulin. We have no explanation for this *in vitro* effect of these iodo-compounds at present and this is currently under investigation.

This anomalous behaviour for the 5-iodo-series of drugs seems unlikely to have any significance because we have been unable to elicit similar behaviour in experiments employing the iodo-series of drugs to inhibit DNA synthesis in cultured cells [23]. In this latter study [23] we found the 5-iodo-series of drugs to be more potent than the parent compound W7 in inhibiting proliferation of K562 tumour cells cultured in serum-free media. Closer examination of 5-iodo-1-C_8 (e) revealed a similar time course of inhibition to W7 and that, as with W7, growth was arrested by inhibiting entry of cells into S phase.

Specificity of action of naphthalenesulphonamides as calmodulin antagonists

Based on the potency data for this series of naphthalenesulphonamides, the C-6 and C-8 side chain 5-iodo-compounds, 5-iodo-1-C_6 (c) and 5-iodo-1-C_8 (e), were selected for further examination to compare their selectivity for non-calmodulin enzyme inhibition with that of W7. The question to be addressed was whether an improvement in calmodulin inhibitory potency was associated with any change in specificity of action of these drugs for calmodulin-dependent versus calmodulin-independent, but calcium-dependent, enzyme processes. Protein kinase C and transglutaminase (EC 2.3.2.13) were selected as good examples of calcium-dependent but calmodulin-independent enzymes [24, 25]. The results of this comparison are shown in Table 2 and Fig. 3.

5-Iodo-1-C_6 (c) is slightly more selective than W7 for calmodulin-dependent enzyme inhibition and 5-iodo-1-C_8 (e) is significantly more selective. Approximately 300 times more 5-iodo-1-C_8 is required for 50% inhibition of protein kinase C or transglutaminase than for inhibition of calmodulin-dependent phosphodiesterase. This compares to a selectivity factor in our hands of only 10-fold for W7 (Table 2 and Fig. 3). This clearly illustrates that improvements in potency of the naphthalenesulphonamides can be concomitant with improvements in specificity of action *in vitro*. Several other structurally different naphthalenesulphonamides and trifluoperazine are illustrated in Table 2 for comparison. *N*-Dansylcadaverine and *N*-TIPS cadaverine are compounds which show reasonable potency in inhibiting transglutaminase but the latter of these is, unfortunately, equipotent in inhibiting calmodulin-dependent phosphodiesterase. Trifluoperazine, like W7, shows only a factor of 10-fold separating its potency against protein kinase C [6] from that of its potency against phosphodiesterase. ^{3}CF-1-C_6 (p) shows little inhibitory effect on any enzyme activity. Comparison of the data in Table 2 indicate that the selectivity achieved for 5-iodo-1-C_8 is without parallel.

Despite its great importance, there are relatively few reports on the specificity of action of calmodulin antagonists. Some drugs, such as R24571 (calmidazolium), a derivative of the antimycotic agent miconazole, despite great potency as calmodulin antagonists (IC_{50} values of 0.03 μM in our own laboratory (see Table 2) and 0.1 μM elsewhere [4] for calmodulin-dependent phosphodiesterase), have been found to have relatively little specificity. Mazzei *et al.* [4] in a thorough study of calmidazolium found that it would also inhibit protein kinase C at relatively low concentrations (IC_{50} of 5.3 μM). The possibility also exists of course that any of the anti-calmodulin drugs may inhibit nonidentified calcium-independent enzymes *in vivo*.

In this study we have shown that it is possible, at least *in vitro*, to improve the potency at the same time as improving the selectivity of action of naphthalenesulphonamide drugs for the inhibition of calmodulin-dependent enzyme activity. The potential value of these new drugs as pharmacological agents for the study of calmodulin biochemistry and as possible clinical tools in carcinostasis and drug resistance is under investigation.

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