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RATIONAL DESIGN OF SELECTIVE LIGANDS FOR TRYPANOTHIONE REDUCTASE FROM *TRYPANOSOMA CRUZI*. STRUCTURAL EFFECTS ON THE INHIBITION BY DIBENZAZEPINES BASED ON IMIPRAMINE

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Trypanothione reductase, the enzyme which in trypanosomal and leishmanial parasites catalyses the reduction of trypanothione disulphide to the redox-protective dithiol and has been identified as a potential target for rational antiparasite drug design, has been found to be strongly inhibited by tricyclic compounds containing the saturated dibenzazepine (imipramine) nucleus, with K_i values in the low micromolar range. This drug lead structure was designed by molecular graphics analysis of a three-dimensional homology model, focussing on the active-site. Inhibition studies were carried out to determine the effect of inhibitor structure on the inhibitory strength towards recombinant trypanothione reductase from *Trypanosoma cruzi*. Hansch analysis showed that inhibitory strength depended on terms in π, π^2 and σ_m indicating dependence on both lipophilicity and inductive effect for ring-substituted analogues of imipramine. The side-chain ω-aminoalkyl chain had to be longer than 2-carbon units for inhibition. The effect on inhibition strength of the substituent at the ω-amino position on the side-chain of the central ring nitrogen atom depended markedly on the detailed substitution pattern of the rest of the molecule. This provides kinetic evidence studies of multiple binding modes within a single, blanket binding site for the inhibitor with the tricyclic ring system in the general region of the hydrophobic pocket lined by Trp21, Tyr110, Met113 and Phe114. This aspect of the structural sensitivity of the precise active-site triangulation adopted by the inhibitor is probably a function of the use of hydrophobic interactions of low directional specificity

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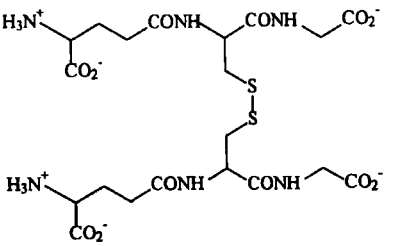
Abbreviations: dmapa – 3-*N*,*N*-dimethylaminopropylamide; GR – glutathione reductase (EC 1.6.4.2); TR – trypanothione reductase (EC 1.6.4.8); $T[S]_2$ – oxidised trypanothione; $T[SH]_2$ – trypanothione as dithiol; GSH – reduced glutathione; GSSG – glutathione disulphide.

in this pocket combined with an electrostatic anchoring by the ω-N^+HMe_2 function of the inhibitor, presumably with a glutamate side-chain, such as Glu-18, Glu-466′ and/or Glu-467′.

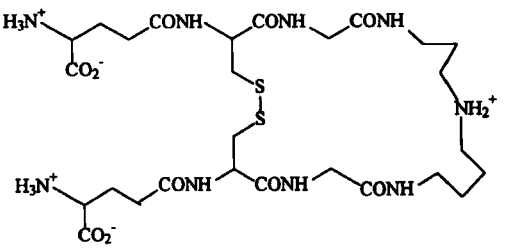
Keywords: Tricyclics; Trypanosomiasis; Rational drug design; Antiparasitic drugs; Leishmaniasis

INTRODUCTION

Diseases caused by trypanosomes and leishmanias, such as African sleeping sickness and Chagas' disease of S. America, annually cause many thousands of fatalities. Each year new victims fall to these diseases, but the chemotherapeutic treatments available, limited in scope and efficiency, are frequently accompanied by severe, sometimes limiting, side-effects. Hopes of a rational means to drug design against this problem rose when it was found[1] that the redox defences of these parasites depend, not on glutathione (GSH) as do those of the host organisms, but on the related molecule trypanothione, converted during this role to a disulphide ($T[S]_2$, Scheme I) much as GSH is converted to glutathione disulphide. The enzyme in these parasites which reduces $T[S]_2$ back to the active dithiol form ($T[SH]_2$) is trypanothione reductase (EC 1.6.4.8). Whilst it carries



Glutathione disulphide **GSSG**



Trypanothione disulphide **T[S]2**

SCHEME I

out a reaction chemically similar to that of the analogous host enzyme (glutathione reductase, EC 1.6.4.2) and has many structural and catalytic features in common with it (such as coenzyme usage and binding) there is almost total mutual substrate exclusivity shown by glutathione reductase (GR) and trypanothione reductase (TR).[2] Thus, the natural substrates can distinguish between host GR and parasite TR, and alternative substrates have been developed to be specific for TR over GR by replacement of the spermidine of $T[S]_2$ by a glycyl-3-dimethylaminopropylamide group.[3,4] Analysis of the molecular determinants of the active-site of TR discriminating between GSSG and $T[S]_2$ showed that the major difference is the presence of a hydrophobic pocket in TR, known to bind one of spermidine moieties of a second physiological substrate, glutathionylspermidine disulphide, and therefore presumed to bind to the spermidine portion of $T[S]_2$.[5] This pocket is formed by Trp21, Tyr110, Met113 and Phe114,[6,7] a region which in GR contains positively charged residues to locate the glycyl carboxylate ions of the GSSG substrate.[8,9]

By molecular graphics analysis of a homology model of TR from *T. congolense* we predicted certain families of tricyclic antidepressants to be selective inhibitors of TR relative to glutathione reductase, and they were found to bind to the former at pH 7.25 with K_i values in the micromolar range.[10] X-ray coordinate data are now available for TR, including crystal forms with substrates or coenzymes bound[5–7,11,12] and recently with mepacrine bound.[13] We now present a more detailed analysis of the inhibition of recombinant TR from *T. cruzi* by saturated dibenzazepines of the imipramine family (**1** to **26**) including molecular graphics and quantitative structure–activity (QSAR) analysis of the inhibition.

MATERIALS AND METHODS

Enzyme Assays

Trypanothione reductase from *T. cruzi* was isolated by means of overexpression of the gene in *E. coli* JM109 cells bearing the expression vector pBSTNAV[14] as previously described.[10] The enzyme, homogenous by the criterion of SDS PAGE, had a specific activity identical to wild-type TR.[15] Enzyme activity was assayed at 25°C in 0.02 M HEPES buffer, pH 7.25, containing 0.15 M KCl, 1 mM EDTA, 0.12 mM $T[S]_2$ and 0.1 mM NADPH[15] at an enzyme concentration of approximately 0.3 $\mu g\,ml^{-1}$. Human erythrocyte GR was isolated from human erythrocytes as described[16] and assayed following literature conditions.[17]

Molecular Graphics

Initial inhibitor design was guided by a computer graphics model of *T. congolense* TR constructed in this laboratory[18] by modification of the published coordinates of human erythrocyte GR.[8] The design process focused on the $T[S]_2$-binding site. Potential inhibitors were designed to bind via specific interactions, namely charge–charge and hydrophobic, to the enzyme. Structures were constructed using the CHEMNOTE function of QUANTA. Energy minimisation was performed using CHARMM. Towards the end of the project, the *C. fasciculata*[5–7] and *T. cruzi*[11] crystal structures were reported and the *C. fasciculata* coordinates used to replace the modelled coordinates and to rationalise the results obtained.

Inhibition Studies

Inhibition type was assessed by analysing the patterns of three diagnostic classes of plot: $1/v$ versus $1/[S_0]$ for various $[I]$; $1/v$ versus $[I]$ for various $[S_0]$; and $[S_0]/v$ versus $[I]$ at various $[S_0]$. Values of K_i were determined by direct weighted ($1/v^2$ for weighting) least squares nonlinear regression analysis of the raw data using the equation for linear competitive inhibition ($v = V_{max}[S_0]/([S_0] + K_m(1 + [I]/K_i))$) using the SIPHAR programme, distributed by SIMED. Values of V_{max} and K_m were obtained by least squares nonlinear regression analysis using the Grafit programme (distributed by Sigma Chemical, Co.) and I_{50} values were determined by fitting data to the appropriately rearranged form of the equation for linear competitive inhibition which was then written into the Grafit programme for nonlinear regression analysis. Quantitative structure–activity relationships (QSAR) were assessed using the QSAR-PC : PAR regression package (distributed by Elsevier Biosoft). Physicochemical substituent constants (π, σ_m, MR) used for compounds **2–10** were from Hansch.[19] Reversibility of enzyme inhibition was assessed using dialysis.

RESULTS

Comparison of the crystallographic *C. fasciculata* and modelled *T. congolense* co-ordinates showed that the overall match was good, but that there were two regions of low correspondence, both surface loops. The model was clearly predictively useful as, prior to availability of X-ray data, it had been used to generate both tricyclic[10] and peptide[20] leads for novel

inhibitors. However, there may be an element of good fortune in this because, although side-chain co-ordinates for modelled and X-ray structures corresponded well for the active-site region, the OH group of Y110 was displaced sufficiently (approx. 1.5 Å in the modelled structure relative to the X-ray) that rational design of ligand interactions to make use of its phenolic OH group would have been severely compromised.

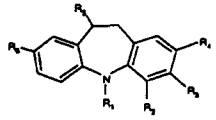
The tricyclics based on the dibenzazepine nucleus were found to be inhibitors of TR with the I_{50} values, the concentrations of inhibitor which lower the activity of the uninhibited enzyme by one-half, shown in Table I. Detailed kinetic studies were performed for a number of compounds to determine inhibition type and K_i values. Thus imipramine was found to be a linear, competitive inhibitor of TR, by the criteria described in the Materials and Methods section, with $T[S]_2$ (or in some cases the alternative substrate, *N*-benzyloxycarbonyl-L-cysteinylglycyl-3-dimethylaminopropylamide disulphide[4]) as variable substrate. For visualisation the set of diagnostic plots for imipramine with $T[S]_2$ as variable substrate is shown as Figures 1A–1C. For statistical reasons the value of K_i was calculated using SIPHAR, not from the linear plots used for display and diagnosis. The effect of changing the structure of the central ring system on inhibition was studied by determining I_{50} values (and K_i values for **30–32**). Data are summarised in Table II.

To test the reversibility of the inhibition, four 1 ml assays were performed, two control and two with clomipramine (29 μM) present. The reaction mixtures were allowed to incubate until substrate processing was complete and then dialysed against assay buffer (0.02 M HEPES buffer pH 7.25, 4°C, containing 0.15 M KCl, 1 mM EDTA) for 16 h with 2 changes. Assays were then repeated using an aliquot (500 μl) of reaction mixture to which DTT (0.1 mM) and NADPH (0.1 mM) had been added, the reaction being initiated by $T[S]_2$ (0.23 mM). Before dialysis, the initial velocity was decreased by 66% in the presence of clomipramine. After dialysis, the initial velocities for all four reaction mixtures were the same to within experimental error. Thus, the inhibition of *T. cruzi* TR by clomipramine is fully reversible.

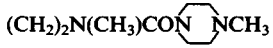
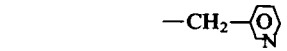
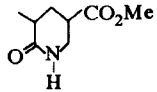
DISCUSSION

Data are collected in Table I for tricyclic compounds related to imipramine with a range of ring substituents and with the side-chain on the ring nitrogen varying with respect to chain length, branching and aromatic

TABLE I Inhibition data for saturated dibenzazephine tricyclic compounds of the imipramine family using *T. cruzi* TR and human erythrocyte GR. For I_{50} determinations the concentrations of trypanothione and NADPH were 0.12 mM and 0.1 mM, respectively



Compound	R_1	R_2	R_3	R_4	R_5	R_6	*TR Inhibition* I_{50} (μM)	*Highest Conc. Used with GR**
1	H	H	H	H	H	H	None at 0.1 mM	0.1 mM
2 (Imipramine)	$(CH_2)_3N(CH_3)_2$	H	H	H	H	H	180 ± 35	1.0 mM
3	$(CH_2)_3N(CH_3)COCH_3$	H	H	H	H	H	2530 ± 370	1.0 mM
4 (Clomipramine)	$(CH_2)_3N(CH_3)_2$	H	Cl	H	H	H	32.4 ± 4.4	1.0 mM
5	$(CH_2)_3N(CH_3)_2$	H	CF_3	H	H	H	55.7 ± 6.1	1.0 mM
6	$(CH_2)_3N(CH_3)_2$	H	Br	H	H	H	66.9 ± 5.2	1.0 mM
7	$(CH_2)_3N(CH_3)_2$	H	NH_2	H	H	H	637 ± 61	Not determined
8	$(CH_2)_3N(CH_3)_2$	H	$NHCH_3$	H	H	H	1240 ± 180	1.0 mM
9	$(CH_2)_3N(CH_3)_2$	H	$N(CH_3)_2$	H	H	H	1010 ± 110	0.1 mM
10	$(CH_2)_3N(CH_3)_2$	H	NO_2	H	H	H	201 ± 33	Not determined
11	$(CH_2)_3N(CH_3)_2$	H	CN	H	H	H	32.2 ± 5.5	1.0 mM
12 (Trimipramine)	$CH_2CHCH_3CH_2N(CH_3)_2$	H	H	H	H	H	297 ± 59	1.0 mM
13	$(CH_2)_2N(CH_3)_2$	H	Cl	H	H	H	115 ± 9	1.0 mM
14	$(CH_2)_3N(CH_3)_2$	H	SO_2N	H	H	H	574 ± 76	
15	$(CH_2)_3N(CH_3)SO_3H$	H	H	SO_3H	H	H	792 ± 110	
16	$CH_2CH(CH_3)CH_2NHCH_3$	H	H	H	H	H	80.1 ± 4.8	

17	$(CH_2)_2N(CH_3)CO$ 	H	H	H	H	H	715 ± 64	0.2 mM
18		H	H	H	H	H	480	0.1 mM
19	$(CH_2)_3N(CH_3)_2$	NO_2	H	H	H	H	580	0.1 mM
20	CH_3		H	H	H	H	2500	0.2 mM
21	$(CH_2)_3NHCH_3$	H	H	OCH_3	H	H	1230 ± 90	
22	CH_3	H	H	H	$(CH_2)_2NHCH_3$	H	1000	1.0 mM
23	$(CH_2)_3N(CH_3)_2$	H	Cl	OH	H	H	200 ± 11	1.0 mM
24	$(CH_2)_3NHCH_3$	H	Cl	OH	H	H	2100	0.5 mM
25	$(CH_2)_3N(CH_3)_2$	H	Cl	H	H	OH	107 ± 14	1.0 mM
26	$(CH_2)_3NHCH_3$	H	Cl	H	H	OH	158 ± 68	1.0 mM
27	$(CH_2)_2NHCH_3$	H	Cl	H	H	H	185 ± 15	1.0 mM
28	$(CH_2)_3N(CH_3)CH_2CH_2OH$	H	Cl	H	H	H	89.1 ± 14.9	1.0 mM
29	$(CH_2)_3NHCH_3$	H	$N(CH_3)_2$	H	H	H	643 ± 83	1.0 mM
30	CH_2CONH_2	H	H	H	H	H	4130 ± 160	0.5 mM

*No inhibition of GR was detected at this concentration of compound.

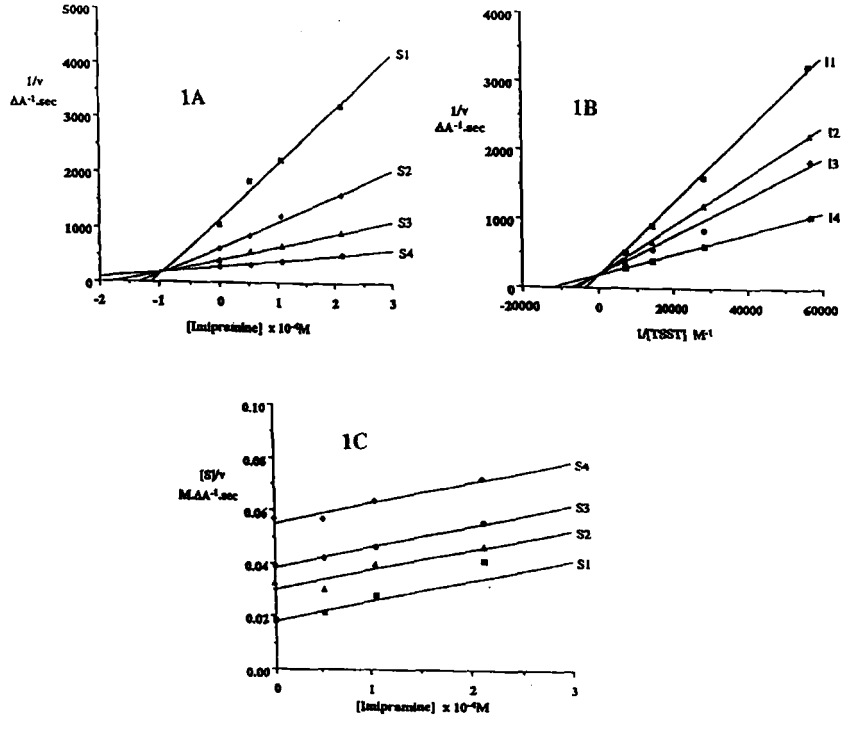
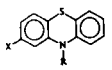
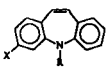
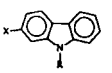
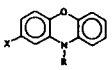
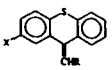
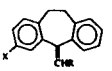
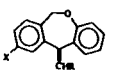
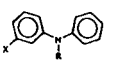


FIGURE 1 Diagnostic plots of inhibition type for imipramine with trypanothione reductase (0.3 μg/ml) from *T. cruzi* studied at 25.0°C in 0.02 M HEPES buffer at pH 7.25 containing 0.15 M KCl and 1 mM EDTA and in the presence of 0.1 mM NADPH. Points are experimental: lines are theoretical assuming linear competitive inhibition with values of $V_{max} = 7.7 \times 10^{-3}\,\Delta A\,sec^{-1}$, $K_m = 52.4\,\mu M$ and $K_i = 96\,\mu M$. (A) Dixon plot using trypanothione as substrate at the following concentrations: $S_1 = 17.5\,\mu M$, $S_2 = 35\,\mu M$, $S_3 = 70\,\mu M$ and $S_4 = 140\,\mu M$. (B) Lineweaver–Burk plot of the data in Figure 1A at the following inhibitor concentrations: $I1 = 213\,\mu M$, $I2 = 107\,\mu M$, $I3 = 53\,\mu M$ and $I4 = 0\,\mu M$. (C) Cornish–Bowden plot of the data in Figure 1A at the same substrate concentrations as indicated in Figure 1A.

content. The unsubstituted parent ring structure (**1**) was found to be inactive as a TR (or GR) inhibitor at 0.1 mM (studies were hampered by poor solubility). Carbamazepine, a related dibenzazepine compound with side-chain $-CONH_2$ equivalent to position R_1, was also inactive at 1.0 mM. Most compounds in Table I are substituted at position R_1. However, compounds with the side-chain at an alternative position still exhibited broadly comparable TR inhibition, compare **22** with **27**. They differ in both the positioning of (*N*-methylamino)ethyl side-chain and the presence in **27** of a ring chloro substituent. The effect of chloro-substitution at position R_3 can be estimated by comparing **2** and **4** (which both have (*N*,*N*-dimethylamino)propyl substituents) and in this case the effect of Cl is to lower the

TABLE II Inhibition data at 25°C in pH 7.25 0.02 M HEPES buffer containing 0.15 M KCl and 1 mM EDTA for tricyclic compounds with varying central ring structure with trypanothione reductase from *T. cruzi* showing I_{50} values (with K_i values in parentheses where determined). For all compounds for which K_i values were determined, the inhibition was found to be linear competitive with respect to trypanothione disulphide. For I_{50} determinations the concentrations of trypanothione and NADPH used were 120 μM and 100 μM, respectively, at an enzyme concentration of approximately 0.3 μg ml^{-1}

Nucleus	*Compound Number*	R	X	*TR Inhibition* $I_{50}(K_i)$, μM
	**30**	$(CH_2)_2N(CH_3)_2$	H	130(55)
	31	$(CH_2)_3N(CH_3)_2$	Cl	400(100)
	32	$CH_2CH(CH_3)CH_2N(CH_3)_2$	H	250(120)
	**33**	$CH_2CH(CH_3)CH_2N(CH_3)_2$	Cl	200
	34	$(CH_2)_3NHCH_3$	CF_3	43.2 ± 3.5
	35	CH_2CONH_2	H	2840 ± 140
	36	$(CH_2)_3NMe_2$	SO_2NMe_2	728 ± 82
	**37**	$(CH_2)_3N(CH_3)_2$	H	776 ± 228
	**38**	$(CH_2)_3N(CH_3)_2$	H	1460 ± 320
	**39**	$(CH_2)_2N(CH_3)_2$	Cl	200
	**40**	$(CH_2)_2N(CH_3)_2$	H	208 ± 27
	41	$(CH_2)_2NHCH_3$	H	211 ± 16
	**42**	$(CH_2)_2N(CH_3)_2$	H	270 ± 78
	**43**	$(CH_2)_3N(CH_3)_2$	H	581 ± 52
	44	$(CH_2)_3N(CH_3)_2$	Cl	155 ± 19

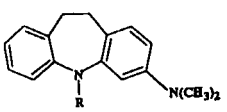
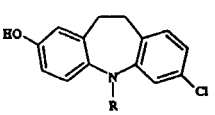
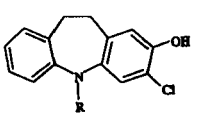
I_{50} value by 6-fold, consistent with the 5.9-fold difference for **22** and **27** arising largely from the Cl substituent.

The lowest I_{50} values were obtained with the side-chain $-(CH_2)_3N(CH_3)_2$ at position R_1 (e.g. imipramine (**2**), $I_{50} = 1.8 \times 10^{-4}$ M and clomipramine (**4**), $I_{50} = 3.2 \times 10^{-5}$ M). Shortening the chain length by one methylene unit increased the I_{50} value (compare (**13**) $I_{50} = 1.15 \times 10^{-4}$ M and (**4**) $I_{50} = 3.2 \times 10^{-5}$ M). Branching the chain by a methyl group (**12**, relative to unbranched imipramine) did not significantly alter the K_i values. This compound contains a chiral centre and hence could have been a racemic mixture (the manufacturer could not specify). Longer or more complex side-chains (consider **16**, **17**) can also be accommodated by the enzyme. However, the substitution of the ω-nitrogen of the side-chain is

important to inhibitory strength. Replacing one of the NMe_2 group's methyl substituents by CH_2CH_2OH (**28**) weakens inhibition by 2-fold, compared with imipramine.

An important feature of substituent effects in this family of TR inhibitors emerges if one compares the effect of changing the substituent on the central ring nitrogen atom from *N*,*N*-dimethylaminopropyl to *N*-methylaminopropyl (Table III). Depending on the structure of the rest of the molecule, this constant change at the ω-amino function can weaken inhibition by as much as 10-fold (cf. **23** and **24**), leave it essentially unaltered (cf. **25** and **26**) or even slightly strengthen it (cf. **9** with **29**). The most likely explanation for this is that the members of this family of TR ligands can adopt a number of binding modes in a generalised binding site and that the precise triangulation of the tricyclic molecule with respect to its TR docking site depends on its detailed substituent pattern. This may result from the tricyclic nucleus binding in the region of the hydrophobic wall (formed by Leu 17, Trp 21, Tyr 110, Met 113, Phe 114). Molecular modelling shows that this hydrophobic pocket is actually slightly larger than the tricyclic nucleus and so the inhibitor could bind at slightly different angles to it to optimise hydrophobic contacts as well as the electronic distribution (effected by ring substitution, and discussed later). A slight angular change

TABLE III Effect of aromatic ring substitution patterns on the relative inhibitory strength towards trypanothione reductase from *T. cruzi* at pH 7.25 m HEPES buffer

Nucleus	$I_{50} \times 10^{-4}$(M)*		$I_{50}\frac{N(CH_3)_2}{NHCH_3}$
	$R=(CH_2)_3N(CH_3)_2$	$R=(CH_2)_3NHCH_3$	
	10.1 (**9**)	6.43 (**29**)	1.6
	1.07 (**25**)	1.58 (**26**)	0.7
	2.0 (**23**)	21 (**24**)	0.1

* The identifying number of the compound (see Table I) is given in parentheses after the I_{50} value.

in location of the tricyclic end of the ligand would be transmitted as a change at the *N*-distal region of the propylamide substituent, altering its binding interactions with TR (e.g. with Glu-466′, Glu-467′). A similar result is obtained if the data for the phenothiazine inhibitors of TR are analysed (J. Garforth *et al.* submitted for publication).

The majority of the tricyclic compounds tested had an aromatic ring substituent in the position *meta* to the bridgehead nitrogen (R_3 in Table I). QSAR was used to analyse the effects of the lipophilicity (π), electron-withdrawing ability (σ_m) and other properties of the substituents and their relationship to potency. Hansch analysis was performed for compounds (**2**, **4–11**) which varied only in the nature of the R_3 substituent. Statistically significant linear relationships were found between $\log(1/I_{50})$ and π, and between $\log(1/I_{50})$ and σ_m. No significant correlation was found between $\log(1/I_{50})$ and molar refractivity (MR). The following regression equations were obtained:

$$\log(1/I_{50}) = 0.56(\pm 0.18)\pi + 3.67(\pm 0.12) \quad (n = 9\ r = 0.762\ s = 0.368), \tag{1}$$

$$\log(1/I_{50}) = 0.96(\pm 0.42)\sigma_m + 3.48(\pm 0.17) \quad (n = 9\ r = 0.651\ s = 0.516). \tag{2}$$

Equation (2) is significant only at the 90% level. Introduction of a π^2 term, reflecting an optimum lipophilicity value, yielded equation (3).

$$\log(1/I_{50}) = 0.61(\pm 0.17)\pi + 0.38(\pm 0.25)\pi^2 + 3.50(\pm 0.16); \quad n = 9\ r = 0.834\ s = 0.338. \tag{3}$$

In spite of the higher correlation coefficient the π^2 term was found not to be significant (95%) using the *t*-test, possibly due to all the data points lying on one 'leg' of the parabola, although the *F*-statistic did exceed the tabular value at the 95% confidence level. Combination of π and σ_m parameters produced regression equation (4) with an improved correlation coefficient relative to those of π and σ_m alone. Thus, activity may depend on both the hydrophobic and electronic natures of the substituent.

$$\log(1/I_{50}) = 0.45(\pm 0.16)\pi + 0.64(\pm 0.33)\sigma_m + 3.54(\pm 0.12); \quad (n = 9\ r = 0.863\ s = 0.310). \tag{4}$$

Inclusion of π^2 in this model yielded equation (5), significant at the 95%

level and accounting for 86.7% of the variance observed in $\log(1/I_{50})$:

$$\log(1/I_{50}) = 0.49(\pm 0.13)\pi + 0.39(\pm 0.18)\pi^2 + 0.65(\pm 0.26)\sigma_m + 3.36(\pm 0.13); \quad (n = 9\ r = 0.931\ s = 0.245). \tag{5}$$

None of the compounds in Table I or II were stronger inhibitors than clomipramine.[10] The effects of variation in the central ring structure can be seen in Table II. Replacing the $-CH_2-CH_2-$ bridgehead by $-S-$ (cf. **12** with **32**) does not alter the I_{50} value. However, replacement by $-O-$ (**38**) weakens inhibition about an order of magnitude (compare **2**). Removal of the $-CH_2CH_2-$ bridgehead as in the fluorenyl derivative (**37**) only weakens inhibition by about 4-fold relative to **2**. Altering the equivalent of the centre ring N atom in **2** by replacing it with $\rangle C=CHR$ (as in **40**, amitriptylene) hardly affects inhibition. Comparing **40** and **42** indicates that the $-CH_2-CH_2-$ to $-CH_2-O-$ (doxepin) bridgehead change also has only a minor influence. It is worth noting that even compounds without the tricyclic bridge (Table II, **43**) can provide inhibitors as effective as their conformationally restricted analogues. This is in accord with the report that substituted-2-amino diarylsulphides gave K_i values of 12–2 μM.[21] However, the exactly comparable ring-opening change made with a 2-chloro substituent weakens inhibition by 5-fold (compare **44** with **4**).

In conclusion, it has been established that a side-chain substituent on the nitrogen of the central ring of the tricyclics is essential for TR inhibition activity. Most compounds tested were substituted at the R_1 position although this substituent could be located elsewhere without total loss of activity. The optimum chain is flexible, five units long, aliphatic, unbranched, present at position R_1 and contains a dimethylamino function as the terminal group, i.e. $-(CH_2)_3N(CH_3)_2$.

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