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A COMPARATIVE STUDY OF *IN VIVO* INHIBITION OF MITOCHONDRIAL FUNCTION IN *SACCHAROMYCES CEREVISIAE* BY TRICYCLIC AND OTHER CENTRALLY-ACTING DRUGS

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Abstract—The tricyclic group of compounds and certain other centrally-acting drugs show selective inhibition of mitochondrial activity in intact yeast cells. A comparative account of potency of different drugs in this respect correlates well with clinical potency. Similarity of mode of action of different drugs at the biochemical level is indicated by cross-tolerance of strains and cross-resistance of spontaneous mutants. The yeast is thus a novel method for the screening of drugs and for determining biochemical activity.

THERE are a number of reports that chlorpromazine and certain structurally related compounds are inhibitors of respiration in intact animals cells and isolated mitochondria (see reviews of Desci[1] on imipramine, and Domino *et al.*[2] and Gordon[3] on the phenothiazines). The results presented here give an account of the yeast cell (*S. cerevisiae*) as a system for screening and comparing a number of psychotropic and related drugs for ability to inhibit growth under conditions requiring functionally active mitochondria.

Previous work from this laboratory[4,5] has shown that chlorpromazine and chlorimipramine preferentially inhibit mitochondrial function in the intact yeast cell. It is possible to make this distinction between cellular response and mitochondrial response to drug action in this organism since it is a facultative anaerobe. Thus in a situation where mitochondrial function only is affected by an inhibitor, growth can proceed by a fermentation alone. In the same situation where the substrate is non-fermentable, growth is arrested. Supplementary evidence in these cases is provided by demonstrating inhibition of O_2 uptake (for a detailed account of the yeast mitochondrion see Roodyn and Wilkie[6]). The inhibitory effect alluded to on respiration[4] appears selective for non-fermentable substrates such as glycerol that feed into the citric acid cycle through pyruvate. The inhibition could occur at the level of transport into, or utilization of, this compound by the mitochondrion. It was also found that the degree of sensitivity to the drugs is strain dependent and that in most strains tested, the mitochondrial system was 5–20 times more sensitive than cellular inhibition; that is, it required 5–20 times as much drug to inhibit growth with fermentable than with non-fermentable substrate. Furthermore, spontaneous mutational changes to resistance affecting only the mitochondria were detected and isolated emphasizing the distinction. Although degree of drug tolerance, both at the cellular and mitochondrial levels, is a characteristic feature of each strain, the latter system is very stable in its response whereas the former fluctuates slightly. Thus ability to inhibit respiration is the

main criterion used in the present series of experiments in comparing activity of a number of drugs, particularly those belonging to the class known as the tricyclics. It was of special interest to compare our classification of drug potency based on the yeast system with those from various pharmacological systems and to look for correlations with clinical potency.

Tricyclic drugs

The compounds listed in Table 1 are tricyclic compounds in a general sense and include tranquilisers, anti-depressants and anti-histamines. However, a distinction between these compounds on clinical grounds is not easy to make. For example, some anti-depressants and anti-histamines have tranquilising effects in certain circumstances. From our tests of their inhibitory effects on respiration (that is ability to arrest growth of cells on non-fermentable glycerol medium, YEPG) all of these compounds, with the possible exception of pheniramines, appear to act in a similar if not identical manner at the biochemical level. This conclusion follows from the finding that relative tolerance levels of the 27 haploid strains tested was the same for each drug. Also spontaneous mutants isolated as resistant to one drug (usually chlorimipramine) were cross-resistant to all other drugs tested. This criterion of cross-relationship in tolerance levels of strains and mutants is of primary importance in comparative studies of drug action. In the case of pheniramines, cross-relationship although good was not 100 per cent. Perhaps the presence of a heterocyclic B ring in the molecule alters activity sufficiently for the yeast system to detect it.

The activity of tricyclic drugs is recorded in Table 1 as the minimum inhibitory concentrations required to arrest growth on solid YEPG medium using the sensitive D26 as tester strain. In some cases the corresponding amounts required to inhibit growth on sugar-containing medium (YEPS) are listed for comparison. ID_{50} was also measured for a number of drugs using shake-flask cultures in YEPG. The plot of percent inhibition against drug concentration gave graphs that were sigmoidal in shape with a linear region between 20 and 80 per cent inhibition. In general the correspondence with activity on solid medium was good, although most drugs were slightly more active in liquid medium.

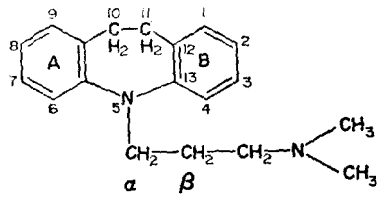
The comparative activity of the drugs allows the following points to be made.

(1) Potency is maximal for desmonomethylimipramine and falls off through imipramine desdimethylimipramine and quaternary imipramine to imipramine *N*-oxide which is inactive.

(2) The $-(CH_2)_3-N<$ chain can be replaced by $-CH_2-CH_2-C(=O)OH$ with good activity but shortening of the side chain to $-C(=O)NH_2$ as in tegratol reduces activity. Total removal of the side chain as in iminodibenzyl abolishes activity.

(3) Substitution at positions 2 or 3 in the ring system appears to correlate less with the electron-donating or withdrawing ability of the group than with its tendency to increase or reduce the hydrophobic nature of the molecule. Thus Cl and CH_3-substituents which tend to increase the hydrophobic nature of the molecule increase potency, while —OH and $-OCH_3$ substituents which tend to make the molecule more hydrophilic reduce potency. Todric and Tait[8] also concluded that no correlation with the degree

TABLE 1. INHIBITION OF CELL GROWTH IN *Saccharomyces cerevisiae* STRAIN D26 BY TRICYCLIC DRUGS IN FERMENTABLE (YEPS) AND NON-FERMENTABLE (YEPG) MEDIUM: COMPARISON WITH INHIBITORY EFFECTS IN OTHER SYSTEMS



Imipramine

Compound	Substitutions in imipramine molecule: Nuclear	Side chain	Terminal N	Inhibitory conc. (M): YEPS	YEPG	Comparative potency: Yeast	ATPase[7]	Rabbit aorta[11]	Metaraminal uptake[29]	Noradrenalin potentiation[9]	Human platelet[8]
Imipramine				6.4×10^{-4}	3.2×10^{-4}	1	1	1	1	1	1
Desmonomethylimipramine			—NHCH$_3$	3.3×10^{-4}	1.6×10^{-4}	2·7	—	7·9	1·2	5·0	0·30
Desdimethylimipramine			—NH$_2$		2.5×10^{-4}	1·3	—	—	—	—	0·25
Imipraminemethachloride			$^+$(CH$_3$)$_3$Cl$^-$		1.1×10^{-3}	0·3	—	—	—	—	3·2
Imipramine *N*-oxide			N(CH$_3$)$_2$		$> 1.5 \times 10^{-3}$	<0·2	—	—	—	—	0·02
2-hydroxy imipramine	—OH (2)				6.4×10^{-4}	0·5	—	—	—	—	0·48
2-methoxydesmethylimipramine	OCH$_3$ (2)		NHCN$_3$		1.1×10^{-3}	0·3	—	—	—	—	—
2,8-dmethylimipramine	CH$_3$ (2,8)				1.0×10^{-4}	3·1	—	—	—	—	—
3,7-diichloroimipramine	Cl (3,7)				4.9×10^{-5}	6·6	—	—	—	—	—
Chlorimipramine	Cl (3)			7.1×10^{-5}	2.8×10^{-5}	10·0	—	—	—	—	5·2
10-methylimipramine	CH$_3$ (10)				2.3×10^{-4}	1·4	—	—	—	—	—
10-oxoimipramine	O (10)				$> 1.5 \times 10^{-3}$	<0·2	—	—	—	—	0·02
Iminodibenzyl *N*-propionic acid		—(CH$_2$)$_2$—COOH as side chain			1.6×10^{-4}	2·7	—	—	—	—	—
α-methylimipramine		CH$_3$ (α)			1.6×10^{-4}	2·7	—	—	—	—	—
β-methylimipramine		CH$_3$ (β)			2.3×10^{-4}	1·4	—	—	—	—	0·02
Iminodibenzyl		no side chain			$> 1.5 \times 10^{-3}$	<0·2	—	—	—	—	—
Opipramol	—CH=CH— (10,11)		—N⟨⟩N (CH$_2$)$_2$OH		1.1×10^{-4}	3·0	—	—	—	—	0·04
Promazine	S-bridge (10,11)			3.2×10^{-4}	1.6×10^{-4}	2·7	0·69	1·05	—	—	—
Chlorpromazine	S-bridge, Cl (3)			1.0×10^{-4}	2.9×10^{-5}	15·0	4·1	1·4	0·72	—	0·07
Prochlorperazine	S-bridge, Cl (3)		—N⟨⟩N—CH$_3$	6.0×10^{-5}	2.4×10^{-5}	18·0	9·5	—	—	—	—
Trifluopromazine	S-bridge, CF$_3$ (3)			1.4×10^{-4}	6.3×10^{-5}	7·0	6·8	—	—	—	—
Chlorprothixene	S-bridge, Cl (3), C for N (5)	C=C (5,α)		1.4×10^{-4}	2.8×10^{-5}	15·0	—	—	0·98	—	—
Trifluoperazine	S-bridge, CF$_3$(3)		—N⟨⟩N—CH$_3$	3.0×10^{-5}	1.0×10^{-5}	43·0	10·5	—	—	—	—
Iprindole	Covalent bridge, 8-carbon B ring			1.6×10^{-4}	3.1×10^{-5}	14·0	—	—	—	0[15]	—
Amitriptyline	C for N (5) C=C (5,α)			1.6×10^{-4}	8.0×10^{-5}	5·4	—	0·42	0·91	0·11	0·54
Nortriptyline	C for N (5) C=C (5,α)		—NHCH$_3$	3.0×10^{-4}	6.5×10^{-5}	6·6	—	3·2	1·05	0·6	0·29
Protriptyline	C for N (5), C=C for bridge		—NHCH$_3$	4.0×10^{-4}	1.7×10^{-4}	2·5	—	7·4	1·2	14·0	0·27
Chlorcyclizine	Cl (2), C for N (5), no bridge at 10,11	—N⟨⟩N-CH$_3$ as side chain		1.5×10^{-4}	7.4×10^{-5}	2·9	—	—	—	—	—
Brompheniramine	No bridge, N for C (13), Br (9)				3.5×10^{-4}	1·2	—	—	—	—	—
Chlorpheniramine	No bridge, N for C (13), Cl (9)			1.3×10^{-4}	6.5×10^{-5}	6·6	—	—	—	—	—

of electropositivity of the substituent in these positions could be found in their system. Substitution of the same group at position 7 as at position 3 does not further increase potency and may reduce it slightly.

(4) Substitution of a methyl group at position 10 in the ring does not greatly affect potency, but 10 oxoimipramine is inactive.

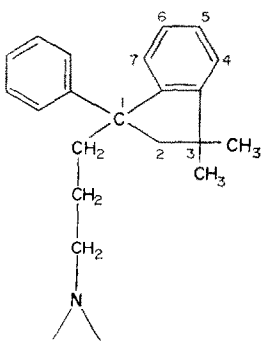
(5) Replacement of the single bond by a double bond between atoms 10–11 (amitriptyline) increases potency, and a carbon atom in place of the nitrogen atom at position five further increases potency (protriptyline).

(6) Replacement of the 10–11 two-carbon bridge by a sulphur atom has by itself a slightly enhancing effect and the substitution of a chlorine atom at position 3 produces chlorpromazine which is more potent than 3-chlorimipramine.

(7) Removal of the bridging atom altogether to give chlorcyclizine produces a highly active compound, but much of the activity is no doubt attributable to the chlorine substituent. Orphenadrine and diphenhydramine, which both lack bridging atoms, and in addition have a side chain containing an ether linkage, possess only weak activity.

Certain compounds obtained through the courtesy of Lundbeck Co. Ltd. which are structurally more distantly related, but which by the criterion of cross-tolerance

TABLE 2. COMPARATIVE POTENCIES OF DERIVATIVES OF 3-3 DIMETHYL PHTHALANE ON CLINICAL TRIAL AS ANTI-DEPRESSANTS (LUNDBECK CO.)



3-3 dimethyl phthalane

Compound serial number	Substitutions in phthalane molecule		Comparative potency (see Table 1)		
	Nuclear	Terminal N<	Yeast	Noradrenalin[8] potentiation	Metaraminal[29] uptake
Lu3-009	O for C (2)	$N(CH_3)_2$	1·0	0·78	1·1
Lu3-010	O for C (2)	$NHCH_3$	2·9	35·0	1·24
Lu3-047		$N(CH_3)_2$	1·5	0·15	—
Lu3-049		$NHCH_3$	4·3	3·2	—
Lu4-074	S for C (2)	$N(CH_3)_2$	3·1	—	—
Lu5-003	S for C (2)	$NHCH_3$	3·6		

probably have the same mode of action as the tricyclics in yeast, were also active (Table 2). These compounds have a similar spectrum of pharmacological activity to the tricyclic anti-depressants.[9,10] Here again potency appeared to correlate best with increasing lipophilicity.

No mention has been made here of a steric interpretation of the results (see Maxwell *et al.*[11]) though this is possible and work along these lines is proceeding.

Several other psychotropic compounds showed selective inhibition of the yeast mitochondrial system, but, by the criterion of cross-tolerance could be differentiated from the tricyclic class. The most interesting of these was haloperidol. This compound has little structural resemblance to the tricyclics but cross-tolerance correlations although weak gave indications of some similarity with the tricyclics. Others in this series included the benzodiazepine compound diazepam (valium) which showed considerable selective activity against yeast respiration but its cogeners oxazepam and chlordiazepoxide (librium) showed little activity. Cross-tolerance studies suggested that the mode of action of diazepam was quite distinct from that of the tricyclics. A possible explanation comes from the observation that another structurally related compound, diazoxide, inhibits succinate oxidation but does not uncouple mitochondria.[12] Amytal also possesses some activity as might be expected from its known action on electron transport[13] and the local anaesthetics procaine and cinchocaine showed activity, cinchocaine considerably more so than procaine. This may be interpreted in the light of the known inhibition of inorganic cation transport in mitochondria by these compounds,[14] the greater activity of cinchocaine being due, presumably, to its greater lipophilicity. Also, as expected, the inhibitor of oxidative phosphorylation oligomycin showed no correlation with the tricyclics although this compound was highly active in its selective inhibition of the yeast mitochondrial system.

Other compounds affecting the central nervous system were tested but showed no activity at the concentrations used (up to 500 μg/ml). These included guanethidine, phenoxybenzamine, α-methyl *m*-tyrosine, amphetamine, ephedrine, reserpine, meprobamate, akineton, methylphenidate, ouabain, chlorthalidone, phenylbutazone, oxyphenbutazone and anturan.

Comparison with pharmacological systems

The comparative potencies of tricyclic drugs are recorded in Table 1 taking imipramine as a standard with a potency of 1. The Lundbeck drugs are recorded in Table 2. This classification was then compared with those recorded for other systems. Published data from various sources are listed and include the inhibitory effects of these drugs on microsomal Na^+-K^+ ATPase,[7] uptake of noradrenaline by rabbit aortic strips,[11] metaraminol uptake by peripheral adrenergic neurons,[10] uptake of 5-hydroxytryptamine in human platelets,[8] and potentiation of noradrenaline response in rats.[9] Our results show some correspondence with those obtained for ATPase inhibition but show little or no correspondence with the other systems which vary widely among themselves. These comparisons serve to illustrate the lack of a pharmacological system for predicting clinical activity. This is particularly striking in the case of the anti-depressant iprindole. A characteristic of anti-depressants is their potentiation of noradrenaline response but iprindole has no apparent activity in this respect either peripherally or centrally.[15] Its inclusion as an active drug in the tricyclic class

from our tests is not in doubt, with a relative potency of 14. The high activity of iprindole may result from the considerable increase in lipophilicity produced by the 8-membered, saturated ring.

Potencies of haloperidol, diazepam, chlordiazepoxide, amytal, procaine, cinchocaine and oligomycin relative to imipramine were respectively 4·3, 1·3, 0·2, 0·2, 0·3, 1·3 and >100.

Comparison with clinical activity

The first point to be noted is that every clinically active drug in the tricyclic group tested was active against the yeast system. Furthermore, changes in clinical potency following the various structural alterations in the basic tricyclic molecule correlate well with the changes recorded in our system. For example, it is well known that the chlorine derivatives if imipramine and promazine are more active clinically than the parent molecules while trifluoroperazine, the most active against the yeast mitochondrion, is perhaps the most clinically active drug in this class. From the point of view of drug screening it would appear that the yeast system is reasonably proficient.

Mechanism(s) of action

There is an increasing amount of evidence that the wide range of actions shown by the tricyclics *in vivo* is due to interaction with membranes[2,16,17] and membrane-bound enzymes.[18,19] The results presented here suggest, not for the first time, that the mitochondrial membrane is the most sensitive membrane.[20]

It is of course interesting to speculate on the exact nature of the inhibitory action on mitochondria, and its possible relation to the pharmacological mode of action of these drugs. It seems from the literature that the precise mode of action of chlorpromazine on mitochondria is not known, and there is evidence that it can have multiple actions depending on the concentrations used. It is also possible that there are differences between the modes of action of chlorpromazine against yeast mitochondria and that against mammalian mitochondria, stemming from differences in electron transport and phosphorylation at site 1 in the electron transport chain, between NAD and flavoprotein,[21] in these two types of cells. It is clear that at appropriate concentrations chlorpromazine can act both as an uncoupler of electron transport and as an inhibitor of phosphorylation. It seems likely however, that inhibition of phosphorylation can occur at concentrations lower than those required to inhibit electron transport. Racker[22] believes chlorpromazine in mammalian mitochondria acts primarily on phosphorylation at site 1, but it is difficult to reconcile this with work suggesting the modification or absence of site 1 in *S. cerevisiae*,[21] the mitochondria of which retain chlorpromazine sensitivity.

The findings published elsewhere[4] that chlorpromazine and chlorimipramine inhibition of pyruvate oxidation can be partially reversed by succinate α-ketoglutarate and certain other citric acid cycle intermediates can be explained in several ways.

(1) That an enzyme, or enzymes involved in pyruvate oxidation specifically are inhibited by these compounds. As citrate synthetase is known not to be inhibited in mammalian mitochondria at least,[23] the membrane-bound pyruvate dehydrogenase complex would seem to be the most likely candidate. The overall effect would be a fall in the amount of reduced pyridine nucleotides available inside the mitochondria and thus a fall in ATP production.

(2) That inhibition of a hypothetical pyruvate transport system[24] is the primary mode of action. This would lead to a deficiency of pyruvate inside the mitochondrion, with subsequent failure of the citric acid cycle and fall-off in ATP production. It is difficult to see how this could account for the immediate inhibition of oxygen uptake that follows the addition of about 1×10^{-4} M chlorpromazine to isolated mitochondria from yeast with pyruvate malate as substrate, or indeed the inhibition caused by larger amounts of chlorpromazine with succinate as substrate. This mode of action could, however, explain the effect of low doses (3×10^{-5} M) of chlorpromazine on whole cell growth, which is cumulative with time.

(3) That the primary effect of chlorpromazine is a direct inhibition of ATP production at one or more of the coupling sites. This provides a likely explanation for the inhibition of oxygen uptake seen with isolated mitochondria in our experiments but it is difficult to see why pyruvate oxidation should be more sensitive than that of other substrates such as α-ketoglutarate which feed in prior to site 1 in the respiratory chain. It could be postulated that the hypothetical pyruvate permease[25] was more sensitive to lack of ATP that other substrate permeases but this seems unlikely.

In the context of these three possibilities it is of interest to note that ATP can protect human cells grown in tissue culture from the lethal effects of chlorpromazine and chlorimipramine.[26] It may also be mentioned that we have shown cross-tolerance between the tricyclics and the organometallic compound triethyltin, suggesting that these compounds have a common mode of action. The significance of this observation is rather reduced by the obscurity surrounding the mode of action of triethyltin. The work of Manger[27] appears to lend some support to hypothesis 2 while the work of Aldridge and Rose[28] suggests that inhibition of phosphorylation is the most important effect of this compound.

As to the relevance of these results to considerations of the pharmacological mode of action of the tricyclics they must strengthen the already strong impression that their action is primarily on membranes, particularly the mitochondrial membrane, and that most of their actions can be explained on this basis. It also seems likely that a mode of action involving limitation of ATP production as a minimum step must be considered for the whole group of compounds despite the diversity of their pharmacological actions. The failure of the yeast system to distinguish between compounds of differing pharmacological activities, particularly to make the distinction between tranquilizers and antidepressants, suggests that this distinction is a property of the cell system in which the drugs operate rather than a difference in biochemical activity of the drugs themselves. Our results so far indicate that limitation of ATP production is the primary step for all the compounds in the tricyclic group. More detailed work on the biochemical aspects is in progress and in which resistant mutants play a significant part.

Techniques described in this paper are the subject of a provisional patent number 18431.

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