Review Article

The Evolution of Antimycobacterial Agents from Non-Antibiotics

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ABSTRACT

The antimicrobial action of a number of non-antibiotic drugs has been demonstrated in recent years, for example the antimycobacterial activity associated with the phenothiazine neuroleptic class. However, as with other classes of non-antibiotics, such activity may be traced back to common precursor structures such as the phenothiazinium dye, methylene blue. While the rising tide of antimicrobial drug resistance has nullified the effects of many conventional agents, several of the original lead compounds and their non-antibiotic derivatives may have a new part to play in the chemotherapy of infectious disease, particularly in the management of tuberculosis.

INTRODUCTION

Among therapeutic approaches, antimicrobial chemotherapy has a relatively short history – around 120 years at the outside, but perhaps more acceptably 75 years since the beginning of clinical sulphonamide use – and derives from much simpler principles. While the use of antibacterials – natural product antibiotics or synthetic-/semi-synthetic agents – is widespread, the huge strides made against infection in the 1940s and 50s are no longer possible, mainly due to the widespread evolution of drug resistance mechanisms among bacteria, in some cases conferring resistance to all known clinical agents. A *prima facie* example of this phenomenon is that of *Mycobacterium tuberculosis*, the principal causative agent in pulmonary tuberculosis that has evolved from multi-drug resistant (MDR, resistance to isoniazid

(INH) and rifampicin (Rif)) to extensively drug resistant (XDR, resistance to INH, Rif, streptomycin, any fluoroquinolone and any of the injectable anti-tuberculosis drugs amikacin, kanamycin and capreomycin) and more recently to total drug resistance (TDR) [1]. In each case, whether MDR/XDR/TDR TB is due to over- or mis-use of the available agents, or required prolonged therapy involving periods of many months - or even an excess of one year as is the situation for therapy of a new case of tuberculosis - or simply because of therapeutic longevity, sufficient selective pressure has been brought to bear, and alternative drugs are in very short supply indeed. This situation is not helped by the single mode-of-action antibacterial approach utilised in the majority of cases. This provides effective selective pressure, it being relatively simple to nullify such drugs in the laboratory via single-point mutation to one member of a drug class, and this may be readily followed by resistance to two or more drugs [2]. Inducement of multi-drug resistance may involve resistance to structurally-similar drugs due to the mutated target [3], or to drugs that are not structurally related [4]. Clearly there is an argument here for the introduction of less-targeted

therapeutics, at least in terms of microbial anatomy. These are currently rare in practice, and multi-site attack relies on therapeutic co-administration. Nor is the parlous state of our antimicrobials the sole concern. Damage to the internal flora following a period of oral administration is often also a cause of illness, and is particularly problematic in the elderly due to the survival and overgrowth of refractory organisms, such as the Gram-positive anaerobe *Clostridium difficile* or the yeast *Candida albicans* [5].

The term *chemotherapy* was coined by the German scientist Paul Ehrlich, relatively late in his highly productive life. Ehrlich's ideas on differential cell staining by aniline dyes during the late 19th and early 20th centuries laid the foundations for what became known as selective toxicity, a principle which still underpins our modern use of antimicrobial drugs. However, Ehrlich's principal contribution to drug discovery was to realise that there was a relationship between dye chemical structure and cell, or organelle, specificity. This quantum leap was manifest in his involvement in the successful clinical treatment of malaria, reported in 1891 [6, 7], for which Ehrlich used the phenothiazinium dye methylene blue (Fig. 1).
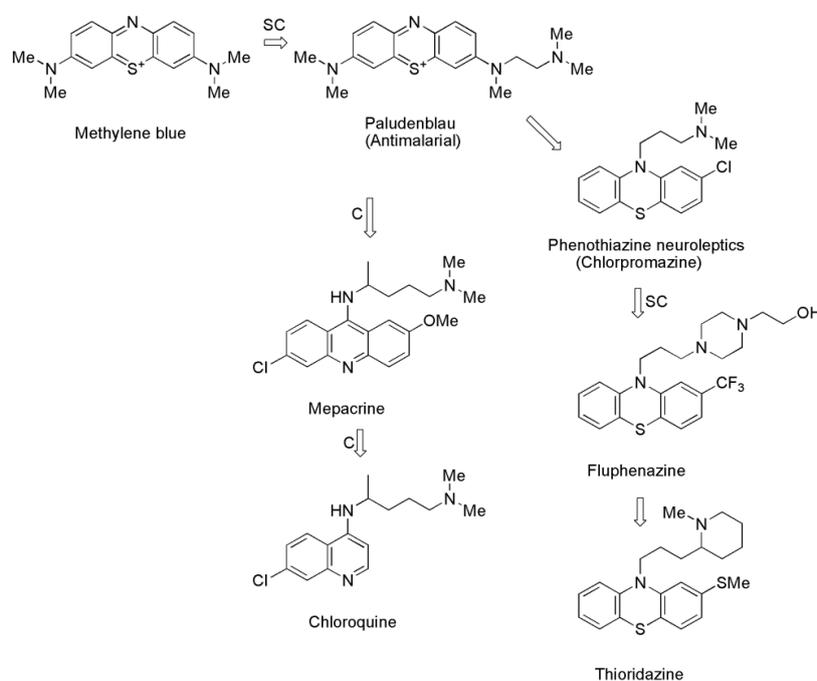

Fig. 1. Drug evolution from methylene blue (phenothiazinium class). Key to significant alterations: C = chromophore change; SC = side chain variation.

The phenothiazine neuroleptics were developed as a result of clinical observations

arising from the use of methylene blue in psychosis as early as 1899 [8-10]. A series of phenothiazines

were then synthesised and tested, those with the alkylaminoalkylamino- side chain (including the ring nitrogen) often exhibiting activity, usually either as H_1 or D_2 antagonists, depending on the length of the side chain, e.g. promethazine and chlorpromazine respectively. Chlorpromazine (CPZ, Fig. 1) became a widely used major tranquiliser for use in the treatment of schizophrenia, and improved derivatives, such as thioridazine (Fig. 1) have been synthesised. In addition, bioisosteric replacement of *N*-10 in the phenothiazine chromophore with trigonal carbon furnished the thioxanthenes, such as flupenthixol, and the alkylaminoalkylamino– side chain can still be seen in more modern D_2/5-HT-active agents such as clozapine and olanzapine (Fig. 2). Similarly, analogue synthesis based on the phenothiazines led to the development of the tricyclic antidepressant class, including imipramine and amitriptyline (Fig. 2).
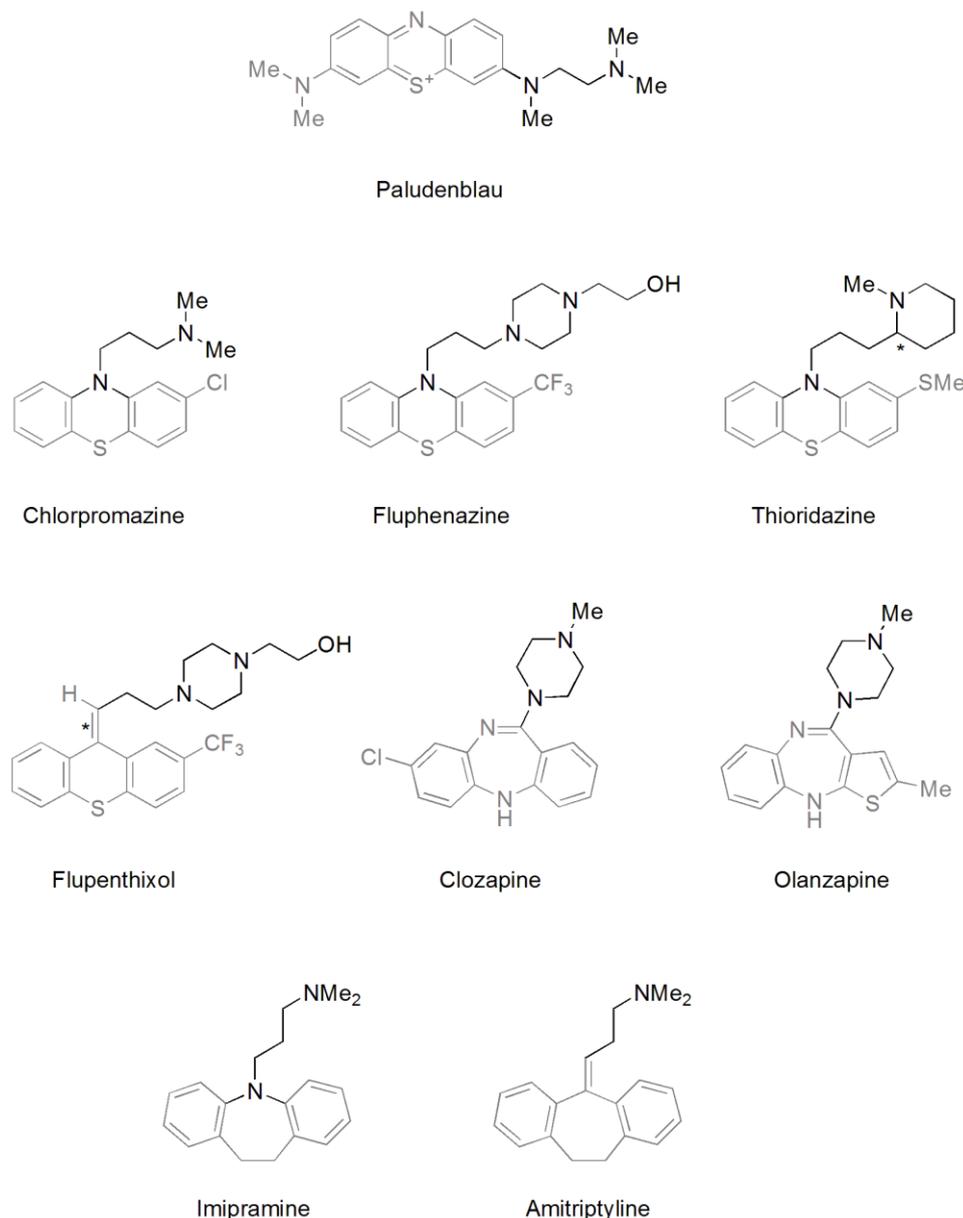
Fig. 2. The alkylaminoalkylamino-type side chain shown in Paludenblau, and emphasised in subsequent neuroleptic molecules (* denotes important stereochemical centre).

The combination of a basic side chain and a tricyclic – normally heterocyclic – chromophore may be observed in each of these therapeutics. In simplistic chemical terms, they represent the same general type of molecule as the early, functionalised methylene blue derivatives

synthesised by Mietzsch and Mauss at Bayer [10] as potential antimicrobial agents (c.f. Paludenblau and modern CNS agents, Fig. 2). While obviously these chemists were not working to a molecular targeting paradigm, as would be expected in modern pharmaceutical research, the compound screening protocols normally included small animals. Thus, organ sequestration/simple pharmacokinetic and toxicity studies would have been possible, in turn allowing a reasonable attempt at the development of rational structure-activity relationships.

Methylene blue is a highly hydrophilic dye. This is reflected in its short half-life in mammalian systems, the greater proportion of the compound appearing rapidly in the urine. What is less well appreciated is the reduction-oxidation ('redox') behaviour of the dye on interaction with the metabolism. Reduced, or 'leuco' methylene blue no longer carries a delocalised positive charge, and exists, at physiological pH, as a neutral molecule. This alters its behaviour in terms of compartmentalisation, serum binding etc. It has also been proposed that the uptake of methylene blue by microbes involves this reduction stage, which facilitates its passage to the cell interior where it may be re-oxidised in the cytoplasm. De Witt in 1913 [11] reported such phenomena in the early years of the last century, having tested methylene blue in a small animal tuberculosis model: grey-coloured tubercles in the post-mortem lung turned blue on exposure to the air.

CNS AGENTS

The early phenothiazine neuroleptic drugs were developed rather empirically – not surprising, given the scant contemporary knowledge of receptor function. The removal of violent symptoms in schizophrenic patients, sufficient for their daily management, was a most persuasive argument for the clinical acceptance of chemical administration. However, the subsequent appearance of serious side-effects was an obvious indicator of a lack of correct selectivity [12, 13].

The development of both the drug chromophore and side chain to this end obviously produced a considerable number of active compounds, covering a wide structural range. As mentioned, the spin-off development of the tricyclic antidepressants may be included here [14]. The whole collection thus represents a significant

number of clinically-acceptable examples of the tricyclic chromophore / basic side chain molecular type.

Although the antimalarial [6, 7, 15, 16] and antibacterial properties of CPZ have been known for decades, due to its frequent serious side effects, it has never been seriously considered for therapy of bacterial infections [17- 22]. However, with the advent of MDR TB infections in the 1980s in areas where TB was thought to have been reduced to the point of eradication, the search for effective anti-MDR TB compounds was begun. Among the compounds examined was Largactil [23, 24], then the commercial preparation of CPZ, which had been shown in the 1950s to cure TB infections and later to have *in vitro* activity against all forms of antibiotic-resistant strains of *M. tuberculosis* [25, 26]. However, the *in vitro* activities occurred at concentrations which were beyond those clinically achievable in serum, but CPZ and other phenothiazines have very high affinity for the lungs, ≤ 100 μg/g wet tissue [27, 28]. At this concentration Kristiansen and Vergmann [26] found that all mycobacteria, independent of resistance pattern, were inhibited by ≤ 25 μg/ml of CPZ on agar. Although this concentration is hundreds of times greater than that safely possible in the human, phenothiazines are known to be concentrated by up to 300% in the tissues, allowing the possibility that the antibacterial concentration might be reached *in vivo*.

These results were confirmed in 1992 and it was also demonstrated that CPZ kills *M. tuberculosis* in newly-phagocytosed human macrophages [29, 30], but interest in CPZ remained low - the CNS side-effects of the agent prevented any serious consideration of its use as an anti-TB drug. Nevertheless, since thioridazine (TZ), the neuroleptic that replaced CPZ, is a much milder drug producing fewer serious side effects than CPZ and was the *in vitro* equal of CPZ with respect to its activity against all forms of antibiotic-resistant strains of *M. tuberculosis* [29], interest in TZ as an anti-MDR TB drug gained credence and it was soon shown to promote the killing of multi-drug resistant strains of *M. tuberculosis* by non-killing macrophages at concentrations in the experimental medium that were below those used in the initial therapy of psychosis [30]. TZ has now been shown to cure both antibiotic-susceptible and MDR TB infection in a murine model [31, 32], and ten out of

twelve cases of XDR TB patients [33]. Protocols have now been published for the therapy of XDR TB whose prognosis is solemn, and may be conducted on a compassionate basis [34].

The widespread use of chlorpromazine during the 1950s also yielded anecdotal observations that the phenothiazine acted synergistically with anticancer therapy [35]. This synergism was later shown to result from the inhibition of the eukaryotic transporter P-glycoprotein (Pgp1) which when over-expressed renders the cancer cell susceptible to the anticancer agent to which it was originally resistant [36]. Similarly, the demonstration that chlorpromazine reduces sensitive and resistant bacteria strains to antibiotics [37, 38] was later interpreted to result from an inhibition of an efflux pump system that extruded the antibiotic prior to its reaching its intended target [39]. Since these studies, phenothiazines have been shown to inhibit the *NorA* efflux pump of *Staphylococcus aureus* [40], the *QAC* efflux pump of the plasmid carried by a *Staphylococcus aureus* multi-drug resistant strain [41], the efflux pump system of E. coli [42], the *AcrAB* efflux pump of E. coli [43, 44], the main efflux pump of Mycobacterium smegmatis [45] and the efflux pump of *Mycobacterium avium* [46]. The phenothiazines chlorpromazine [47] and thioridazine [48] have been shown to affect the activity of genes that regulate and code for the *AcrAB* efflux pump of E. coli as well as that of *Salmonella enteric* serovar Enteritidis [49]. That the activity of thioridazine on bacterial genes is probably universal is illustrated by its effects on the main survival genes of *Mycobacterium tuberculosis* [50].

The mechanism by which this phenothiazine directly affects the activity of an efflux pump has been extensively studied. The effects are indirect and result from the inhibition of calcium binding to calcium-dependent enzymes involved in metabolism [51]. This inhibition reduces the formation of hydronium (H_3O^+) ions that result from metabolism needed for the activity of the pump [52]. Because the dissociation of the substrate from the transporter is pH-dependent – high dissociation constant in low pH and low dissociation constant in high pH [53] - the internal pH of the transporter is not reduced to a level required for dissociation of the substrate as a consequence of reduced availability of hydronium ions [52, 54].

Although nothing has essentially been reported with respect to structural activity relationship (SAR) for thioridazine derivatives that are effective against *Mycobacterium tuberculosis*, a recent study focused on synthesized quaternized chlorpromazine, triflupromazine, and promethazine derivatives, demonstrated that these derivatives had antitubercular activities against both actively growing and non-replicating references wild-type *Mycobacterium tuberculosis* H37Rv [55]. All active compounds were found to have no toxicity against a Vero cell line. Importantly, whereas N-Allylchlorpromazinium bromide rendered the derivatives less active, the replacement of the allyl with benzyl or substituted benzyl promoted significant greater anti-tubercular activity. Moreover, the substitutents that had powerful electron withdrawing properties were essential for the improved activity of the derivatives. All derivatives that contained branching at the carbon chain expressed significantly weaker activity. The main conclusion that can be drawn from these studies is that structures that possessed N-(4- or 3-chlorobenzyl) substitution expressed the highest anti-tubercular activity.

Phenothiazines appear to have other antibacterial effects, causing the elimination of plasmids from Gram-negative [56, 57] and Gram-positive bacteria [58, 59]. Phenothiazines promote the elimination of plasmids from bacteria (plasmid curing) due to the smaller concentration (MIC) of the agent needed to inhibit plasmid replication as opposed to those required for the inhibition of replication of the bacterium. This inhibition results from the intercalation of the agent between nucleic bases of DNA, especially at regions rich in guanosine and cytosine [60]. However, because phenothiazines have significant effects on the cell envelope of bacteria [61, 62] the more facilitated elimination of smaller plasmids as opposed to larger plasmids may be, at least partially due to the size of the plasmid itself [63]. Because the antibiotic resistance of bacterial pathogens relevant to human and animal husbandry is often due to plasmids carrying antibiotic resistance genes [64], the elimination of plasmids from bacteria provides a way to eliminate clinical antibiotic resistance in infection-causing bacteria.

Interestingly, because industry has been told that processes employing bacteria must be conducted with bacteria that have no antibiotic resistance, elimination of plasmids is now being pursued vigorously [65].

During recent years pre-administration of phenothiazines to animals has provided protection from virulent *Salmonella* infections [66, 67]. Because protection takes place with *in vivo* concentrations of the phenothiazine which are 30 times lower than those needed to inhibit the *in vitro* replication of the organism, the mechanism by which protection is afforded is not dependent upon the effects of the agent noted *in vitro*. If the *in vivo* concentration of the agent is so low, how does it protect from an infection by a highly virulent *Salmonella* strain? Moreover, because the mode of infection involved IP administration and subsequent rapid phagocytosis of the bacteria by neutrophils does not result in the killing of the organism, how does the agent protect from infection? The answer may lie in the following: within minutes of phagocytosis and fusion of the lysosome with the phagosome, the low pH of this vacuole induces the activation of two two-component regulons; *PmrA/B* and *PhoP/Q*. The activation of these regulons results in the rapid synthesis of Lipid A which is introduced into the nascent lipopolysaccharide layer of the outer cell wall. Once this occurs the organism is resistant not only to the hydrolytic enzymes of the phagosome-lysosome vacuole, but also to practically everything else [68]. It is postulated that because pre-administration of the phenothiazine results in the accumulation of the agent by lysosome-rich macrophages such as the neutrophil [69, 70], the concentration of the phenothiazine within the lysosome may be sufficiently high to inhibit the first step of the two component regulon-namely, the sensor function of the *PmrA* receptor present on the surface of *Salmonella*. Support for this possibility is provided by the non-specific binding of the phenothiazine chlorpromazine to the surface of the cell blocking access of an O antibody to the O antigen surface of the bacterium [64]. Nevertheless, other possibilities that contribute to the protection provided by the phenothiazine exist in as much as the phenothiazine chlorpromazine has been shown to modulate secretion and syntheses of cytokines involved in protection from infection [71].

THE LIPOPHILIC CHROMOPHORE/BASIC SIDE-CHAIN PARADIGM

In Denmark membrane stabilisers such as anaesthetics, the phenothiazines and chloroquine have been studied in respect to efflux inhibition [72-74]. Kristiansen *et al.* [73-77] have investigated the inhibition of potassium efflux and antimicrobial, immunostimulation and reversal of resistance potency in the same compounds. Interestingly, the observed antimicrobial activities of different phenothiazine and thioxanthene analogues and their metabolites [76-79] are independent of their CNS activity as seen, for example, in the antihistaminic and neuroleptic phenothiazines [24, 80, 81].

It can be emphasized that phenothiazine analogues with an exocyclic double bond (the thioxanthenes) are more potent antimicrobials *in vitro* than the corresponding compounds lacking it [24, 76, 81, 82]. The (Z) isomeric thioxanthenes (Fig. 2) generally exhibit stronger neuroleptic activities compared to the (E) isomers. The possibility therefore exists to separate the CNS and antimicrobial activities by choosing the latter compounds as antimicrobials and increasing this activity by optimising ring substitution using e.g. halogens [74, 76, 77].

Similar behaviour is exhibited by classical phenothiazine compounds exhibiting (+/-) stereochemistry, e.g. promethazine, alimemazine, mepromazine and thioridazine [76, 77, 83, 84]. (+/-) Thioridazine (Fig. 2) is especially important, because this mixture exhibits very potent antimicrobial activity against both resistant (MDR, XDR) and sensitive mycobacterial strains. These compounds are also very active as efflux inhibitors in the reversal of antimicrobial resistance. Unexpectedly, (-)-thioridazine, without CNS activity, is the most potent [77, 83, 84]. This observation constitutes a medical breakthrough for the use of phenothiazines and their analogues, as well as their more active metabolites, together with classical chemotherapeutics for synergy and/or for reversal of resistance *in vivo* [75, 79]. Amaral *et al.* have shown that it is possible to use thioridazine as a "helper compound" in MDR and XMDR TB in *in vitro*, *ex vivo* and *in vivo* investigations [33].

Similarly, Abbate *et al.* have reported successful treatment of seriously ill patients with XDR TB with low doses of thioridazine as a helper compound in a regime together with anti-TB compounds against which the TB strains are resistant [85]. Ehrlich had noted such possibilities in neurotropic compounds, in relation to his theories for developing chemotherapeutics from dye series to be used in serious intracellular infectious disease [75, 86]. Now, a century after Ehrlich, a phenothiazine derivative synthesized in the late 1950s has a role to play in one of the most serious bacterial threats to mankind, XDR TB. The phenothiazines may now have a renaissance as a new antimicrobial class.

The phenothiazines, thioxanthenes and dibenzazepine antidepressants, all obey the lipophilic chromophore/basic side chain paradigm. These lipophilic chemicals and their stereochemical variations have been investigated since late in the 1970s . However, so far there has been much less investigation of the modern antidepressant (SSRI) drugs as efflux inhibitors [37, 87-92] than of the phenothiazines and other, older psychopharmacological agents.

It is perhaps ironic, then, that these less related CNS agents, such as the selective serotonin reuptake inhibitors femoxitine, paroxetine, fluoxetine, sertraline and cipramil and their stereochemical analogues have now been shown to act also as antibacterial, antifungal, antiviral and antiprotozoal compounds both *in vitro* and *in vivo* and as efflux pump inhibitors, exhibiting particular activity against different Gram-positive and Gram-negative bacteria [88-92]. However, on structural consideration, each of these SSRIs can be seen to fulfil the same chromophore/side chain requirement (Fig. 3), and so might also be used as helper compounds in the future as demonstrated by Cecchelli *et al.* in 2010 in the treatment of HIV infected persons [93].
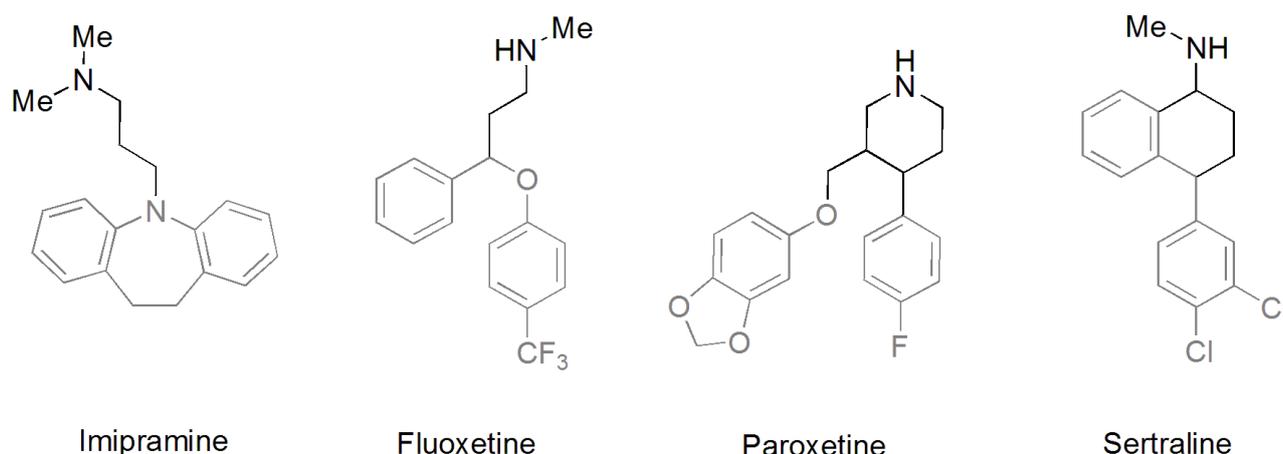

Fig. 3. Imipramine and exemplar SSRI molecules. Chromophore or chromophore-equivalent shown in grey, side-chain/side-chain equivalent in black.

FUTURE CONSIDERATIONS

Because of the urgency required for the therapy of an essentially lethal infection, this review primarily focused on the potential that the phenothiazine thioridazine has for successful therapy MDR/XDR and TDR MT infections. That this potential is real, witness the recently published study that demonstrated again that the use of thioridazine cures the XDR TB [94]. However, it should be noted that in all probability, thioridazine will be useful for the therapy of any intracellular infection such recurrent *Staphylococcal* pulmonary and bone marrow infections [95], as well as Chagas disease, leishmania, and even malaria, given that its precursor phenothiazine chlorpromazine has already been shown to kill the infectious agent *in situ* [96-100].

CONCLUSION

The chemical links demonstrated here between early dyes and modern drugs are

becoming better appreciated by those involved in the pharmaceutical field. In many cases, these should be obvious, given the gross structural similarities in cases such as methylene blue and the phenothiazine CNS agents, but without knowledge of the chemical genealogy involved, they may be less apparent in second- and third-generation agents, such as the SSRIs. In addition, the chemical links do not explain entirely the antimicrobial activities of the non-antibiotic derivatives, although, as noted above, the *in vivo* reduction of methylene blue and its incorporation as the neutral leucobase by tubercles hints at the similarity.

In terms of therapeutic utility, the improvement in selectivity and action of neuroleptic drugs progressing from chlorpromazine to thioridazine and the thioxanthenes allowed effective therapy at lower doses with fewer side effects. A similar pattern has been shown with the antidepressants, from tricyclics to SSRIs. The discovery that many of these agents are also antimicrobial thus offers the prospect particularly of new approaches – and templates for novel drug design - in antibacterial chemotherapy. Given the modern climate of drug-resistant bacteria, this is of considerable importance, and even more so in the area of drug-resistant tuberculosis. The adjunctive use of suitable CNS agents alongside conventional antibacterial agents in order to circumvent clinical resistance is likely to be similarly effective.

Finally, but of no less import, the discovery of antimicrobial effects in drugs/compounds which are already known and in some cases have been in clinical use for many years should provide chemotherapy at much lower cost, thus being of considerable potential in aiding less fortunate health economies.

COMPETING INTERESTS

None to declare.

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