ELSEVIER

Luck, ingenuity and a straightforward chemistry have made phenothiazine the most promiscuous lead structure of the 20th century – and there is more on the horizon.

Foundation review: Phenothiazine: the seven lives of pharmacology's first lead structure

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Rooted in the early days of organic dye chemistry, the phenothiazine structure and its derivatives have since held a prominent place in pharmacology and biomedicine. Initially used for histochemical stains of plasmodia by Paul Ehrlich, anthelmintic and antibiotic properties of phenothiazines were globally exploited in the 1930s and 1940s. Clinical use of *N*-substituted phenothiazines as antihistaminics (1940s), sedatives and antipsychotics (1950s) followed and continues to this day. Recently, interest in these structures has re-emerged for a variety of fascinating features in relation to neurodegenerative disease, spearheaded by the unique redox chemistry of phenothiazine – arguably the most potent chain-breaking antioxidant ever identified.

Introduction

What makes a chemical compound a pharmaceutical lead structure? If it is the recurrence, over more than a century, of medical applications against an ever-expanding spectrum of severe disorders, with all drugs based on the same chemical core, then phenothiazine is probably not only the first but also the most promiscuous lead structure of the 20th century.

Starting their 'career' as putative antimalarials in the late 1880s in the laboratory of one of the most eminent physicians ever – Paul Ehrlich – phenothiazines were globally applied to livestock and humans in the middle of the 20th century – as an anthelmintic saving uncountable lives [1]. In France, a small group of researchers at Rhône-Poulenc Laboratories developed derivatives of phenothiazine with antihistaminic effects, culminating in the introduction in 1950 of promethazine for anesthesia, and many other applications ranging from allergy to seasickness [2]. Shortly afterwards, chemical creativity led to the advent of chlorpromazine, which was administered, for a serendipitously discovered CNS effect, to schizophrenic, agitated and hyperactive psychiatric patients [2]. Antipsychotics with a phenothiazine core were unrivalled for almost 40 years, and they are essential for the clinical treatment of psychiatric disorders to this day. The World Health Organisation (WHO) List of Essential Medicines of 2009 continues to name two phenothiazines: chlorpromazine and fluphenazine, together with haloperidol, as the three indispensable drugs for

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1359-6446/06/$ - see front matter doi:10.1016/j.drudis.2011.01.001

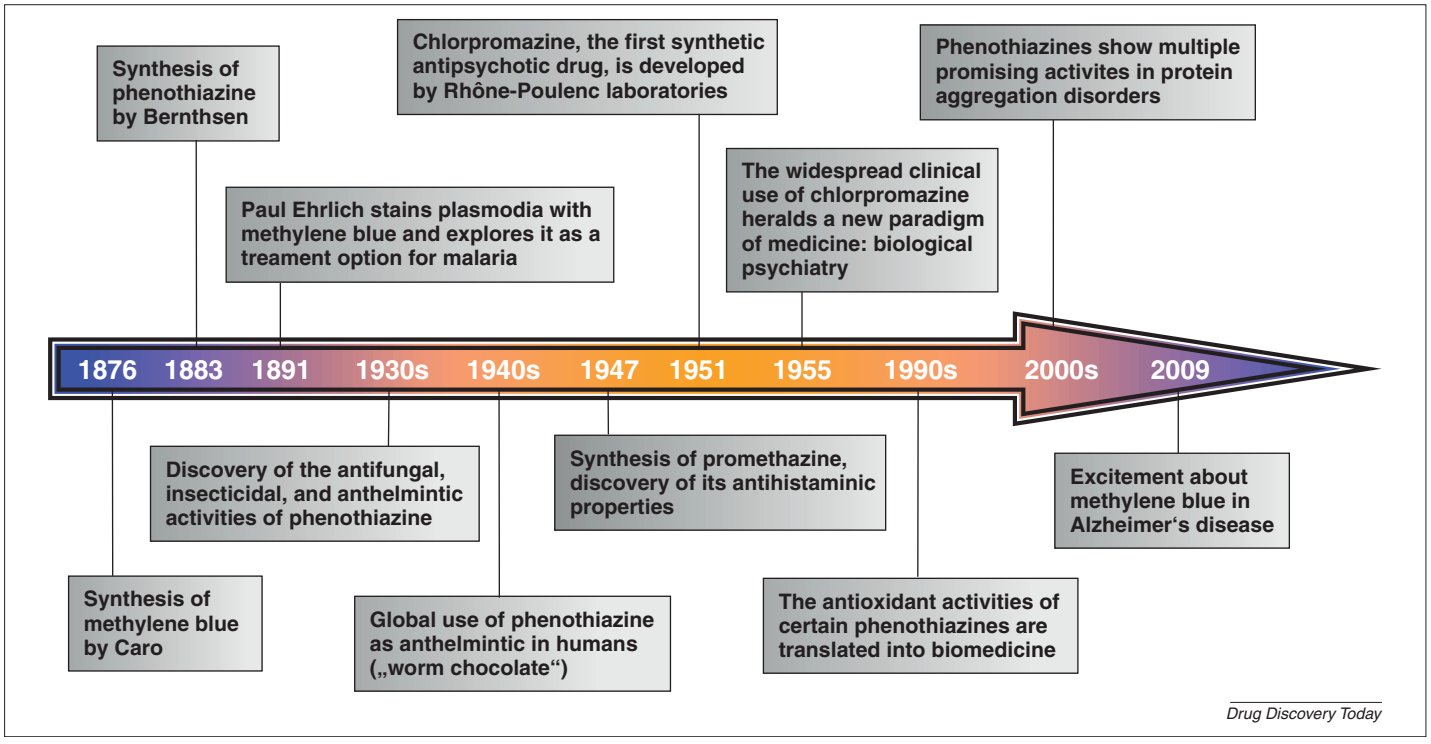

FIGURE 1

Timeline of the most important developments with regard to phenothiazine.

the treatment of psychotic disorders [3]. In fact, the advent of the phenothiazines is widely thought to mark the beginning of biological psychiatry.

The redox activity of phenothiazine and of many congeners had already been described in the 1940s, but primarily related to technical applications in the engineering industry [4]. In the past two decades, with increasing knowledge on the role of oxidative stress in many degenerative human pathologies, interest in redox-active agents and antioxidants as potential therapeutics has recurrently peaked. Because there is growing evidence of a causal involvement of oxidative stress not only in atherosclerosis and diabetes but also in various neurodegenerative disorders, the investigation of phenothiazine, methylene blue and their derivatives in the treatment of Parkinson's and Alzheimer's diseases is probably one of the most fascinating avenues of current-day drug discovery (Fig. 1).

Measure for measure – the inauguration of methylene blue as a histochemical dye

Stimulated by the progress and commercial importance of aniline dyes in the 19th century, the German chemist Heinrich August Bernthsen of Heidelberg started to investigate aniline-based aromatic compounds such as methylene blue **1** to elucidate their chemical structure (Figs. 2 and 3). In 1885, he correctly described the constitution of this dye, two years after he had first synthesized phenothiazine **3** itself by vigorous heating of diphenylamine with elemental sulfur [5]. By 1889, he became head of the core laboratories of the Badische Anilin- und Soda-Fabrik (BASF) that had been co-directed since 1868 by Heinrich Caro, the individual who, in 1876, had first synthesized methylene blue, yet without knowing the constitution of the dye (Fig. 1).

At about the same time in Berlin, Paul Ehrlich had discovered that methylene blue was suitable for blood smear stainings of plasmodia. Based on the simple but revolutionary idea that specific staining equalling specific binding could reflect specific toxicity, he investigated methylene blue as an experimental treatment for plasmodium infection in humans. In 1891 he published his successful therapy of two malaria patients at the Moabit hospital in Berlin [6], whom he had treated with hourly doses of 0.1 g up to a daily cumulative dose of 0.5 g for 8–10 days. In the following decades, methylene blue was frequently administered to patients infected with different species of plasmodia, with rather variable outcomes ranging from fast and relapse-free relief to apparent inefficacy [7]. In the majority of cases, especially regarding the less crucial forms of malaria caused by *Plasmodium malariae* (malaria tertiana) and *Plasmodium ovale* (malaria quartana), methylene blue had a measurable therapeutic effect [7], even if it was noted early that the compound was ultimately not comparable in potency with quinine [8]. Beyond being directly administered to patients, methylene blue was of merit in the battle against malaria because it helped in the development of pamaquine and other aminoquinolines that were to become indispensable for the treatment of malaria tropica during World War II [9].

From the current point of view, it appears that methylene blue is a rather pleiotropic agent that might eventually find its role as a readily available and inexpensive chloroquine sensitizer and inhibitor of *Plasmodium falciparum* glutathione reductase [10,11], even if it is clear that this will not significantly relieve pressure from the pipeline to develop potent new antimalarials [9].

Ehrlich was also the first to describe the metabolism of methylene blue and its peculiar property of redox-cycling (Fig. 2). During

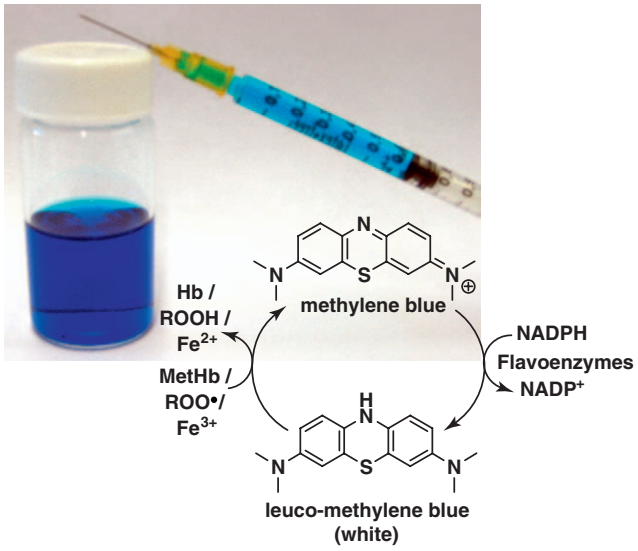

FIGURE 2

Redox cycling of methylene blue. Flavoenzymes reductively convert methylene blue into leuco-methylene blue *in vivo*. The reduced, colourless form can then reduce peroxides, methemoglobin and other cellular targets.

his stainings of different tissues *in vivo* he had seen a variable, reversible reduction of methylene blue to its colourless leuco form **2** in certain organs, for example liver and lung [12]. After exposure to ambient oxygen, the blue colour of oxidized methylene blue returned. With methylene blue, he set out to investigate nervous system anatomy [13], and he also detected its anesthetic activity in small clinical trials [14].

Understandably, public interest into the structural core of methylene blue **1**, the new molecule phenothiazine **3**, did not begin to grow until almost 50 years later, when true health applications for the general public were reported.

The passionate pilgrim – the discovery of a wide spectrum of antimicrobial activities in phenothiazine and the diverse attempts to exploit them

In an early systematic drug screening of sulfur-containing substances for insecticidal activity, Campbell *et al.* noted that phenothiazine itself had a significant effect against culicine mosquito larvae at concentrations of 1 ppm [15]. At about the same time, Howard *et al.* had observed similar effects during their experiments with Mexican bean beetles [16]. Hence, phenothiazine bioactivity was apparently not restricted to single-celled organisms such as plasmodia. For comparison, DDT (dichloro-diphenyl-trichloroethane) was only introduced five years later in 1939 [17]. Many trials with other species of insects were to follow: phenothiazine was found to be rather effective against apple maggot, corn borer, screw worm and silk worm; although less potent against grasshoppers, the Japanese beetle and the tobacco hookworm [1]. Its particularly high efficacy against the apple maggot led to its frequent usage in orchards, where it partly replaced the widely used arsenic formulations (0.2% lead arsenate) [18]. Against different fruit pests the compound was mostly applied as a spray, but the desired insecticidal activity lasted for only a few days, which was generally attributed to insufficient adhesion and fast decomposition of phenothiazine in air and sunlight [19]. Thus, phenothiazine has never become very popular as an insecticide, although a most striking side-effect of its insecticidal use was the observation that accordingly treated fruit showed delayed rotting, which was clearly not attributable to any insecticidal properties, but rather to certain antifungal and antibacterial activities of this compound. In the middle of the 20th century the latter have even been found useful for the clinical treatment of urinary tract infection [20].

Recently, systematic investigations, primarily by Jette Kristiansen *et al.*, have demonstrated considerable antibacterial potency of different phenothiazine derivatives borrowed from psychopharmacology, such as thioridazine or trifluorpromazine, against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus* spp. and, most notably, for the suppression of mycobacterial growth in a manner relevant to tuberculosis [21–23]. Specifically, several phenothiazine derivatives were surprisingly potent agents to sensitize antibiotic-resistant bacteria. For example, in several strains of methicillin-resistant *S. aureus* (MSRA) the minimal inhibitory concentration (MIC value) of oxacillin could be lowered from >256 mg/l down to 1–4 mg/l by the addition of 12 mg/l thioridazine **7**, or down to 4–16 mg/l by the addition of 12 mg/l chlorpromazine **6** [24]. The sensitizers thioridazine and chlorpromazine alone exhibited MIC values of 32 mg/l and 32–64 mg/l, respectively. Similar effects of phenothiazines on antibiotic drug resistance were described for erythromycin-resistant *Streptococcus pyogenes* [24].

Referring to an early case study of a tuberculosis patient treated successfully with the antipsychotic drug chlorpromazine **6** (Fig. 3) [25], Yano *et al.* and Madrid *et al.* have made considerable progress in the elucidation of the possible antitubercular mode of action of substituted phenothiazines that bind to and inhibit NADH-menaquinone oxidoreductase (NDH-2), a pivotal respiratory enzyme for mycobacteria [26]. By means of a systematic substitution strategy, novel phenothiazines could be synthesized with increased antitubercular activity (MIC values of 2–4 mg/l), but with decreased affinity to dopamine and serotonin receptors [27].

Resistance against traditional antibiotics is an emerging medical problem worldwide. The use of readily available, affordable and relatively safe drugs to potentiate the effects of traditional antibiotics or to revert resistance against these, for example, by multidrug efflux pump inhibition in *S. aureus* [28] or independently of multidrug efflux pump inhibition in *Enterococcus* spp. [29], could provide interesting alternatives to the *ab initio* search for, and development of, totally novel compounds. Moreover, an increased knowledge about the involved molecular mechanisms and structure–activity relationships (SAR) could well lead to a renaissance of the phenothiazines as antibacterials.

As you like it – phenothiazine-containing worm chocolate and the large impact of phenothiazine's anthelmintic properties on 20th century medicine

Whereas none of the antimicrobial activities of the parent compound phenothiazine **3** has found widespread exploitation in the clinic, there is one indication for which it became world-famous:

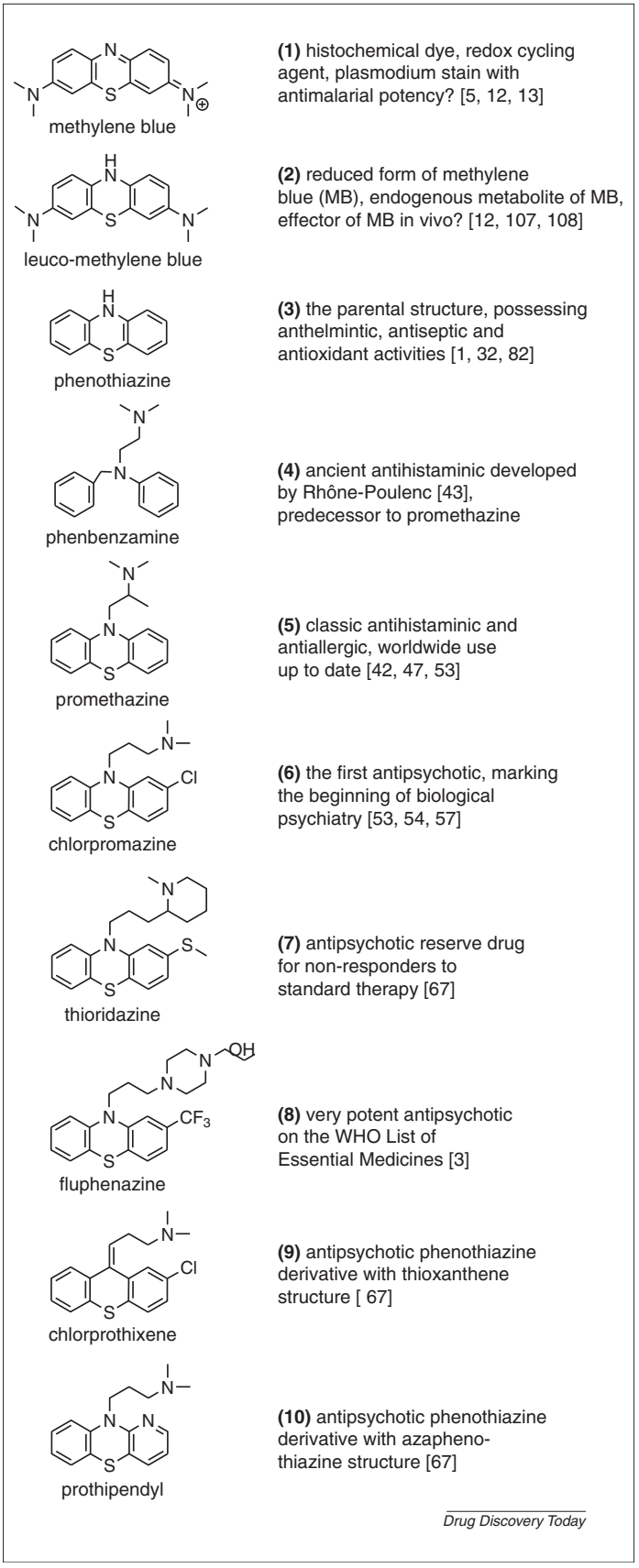

FIGURE 3

Chemical structures of some important phenothiazines and phenothiazine derivatives that have found application as drugs in human pharmacology.

its anthelmintic effect. At the end of the 1930s, veterinarians spearheaded the field and described in diverse publications the oral administration of phenothiazine to pigs, sheep and other livestock. In 1938, Harwood *et al.* showed that the chemical eradicated ascarids and nodular worms from swine [30] after feeding the animals with 1 g phenothiazine per kg body weight. In calves a dose of 0.1 g/kg protected against horn fly infestation by preventing larval development in the manure [31,32]. Hence, the antiparasitic effect of phenothiazine was not restricted to crop pests, it also extended to animal parasites. Within a short time, the considerable power of phenothiazine against intestinal and certain tissue parasitic nematodes led to its use for prophylactic and treatment purposes in the USA, Canada, Great Britain and elsewhere [33] – reflected by a 3000-fold increase in the annual production of the compound between 1939 and 1943 [1].

The anthelmintic application of phenothiazine was not limited to stock farming. By 1940 Philip Manson-Bahr had applied it to human patients with roundworm infections [34]. Children with oxyuriasis (a common threadworm infection in temperate climates) responded especially well to this novel drug and were rapidly cured. Manson-Bahr noted a dose–response effect and gave, for the first time, specific dose and treatment advice [34], distinguishing between age groups. Children under 8 years should get a daily dose of 2 g for 7 days, children under 4 years half of this dose, whereas adults were recommended to take 8 g/d for 5 days. Manson-Bahr was also the first to work on the galenics of phenothiazine, applying it as a paste with syrup [34]. Later on, phenothiazine preparations were usually made with limejuice or chocolate, resulting in what was marketed as 'worm chocolate'. Considering that it was wartime with often limited access to sanitation, antiparasitic and anthelmintic treatments were a major public health benefit. Maybe coming as a surprising spin-off, phenothiazine also helped curing another wartime shortage: that of surgical thread. In the 1940s surgical thread was often made from sheep casings, but casings from parasite-infected animals were too damaged to be used. By eradicating the parasites, it was ultimately phenothiazine that restored this essential medical supply [35].

In view of the considerable doses of phenothiazine that had to be administered to elicit sufficient efficacy, generally >10-fold the doses of modern anthelmintic drugs such as mebendazole or praziquantel, it might not have come as a surprise that Hubble found toxic adverse effects of phenothiazine, the most severe being hemolytic anemia [36]. He had treated a 12-year-old girl, who was sent to him with threadworm infection from the school clinic, with 4 g phenothiazine for seven days. Ensuingly, the girl developed jaundice with insomnia, nausea, anorexia and depression [36]. After seven days the jaundice disappeared, but the child was still anemic, so she got iron administration for one more week and she recovered. Hubble proposed an adaption of dosage to body weight and strict supervision of administration by blood examination [36]. Other groups investigating phenothiazine as an anthelmintic came to similar conclusions [37,38]. In the late 1950s the common use of worm chocolate with 0.2 g phenothiazine per tablet [38] to treat parasitic infection of the intestines was replaced by more potent anthelmintics such as piperazine adipate [39] or the newly developed benzimidazoles, for example thiabendazole (1961).

Given the extensive use of phenothiazine in human pharmacology for more than a decade, it might be viewed as rather surprising that the molecular mechanism of this drug is not known to this date. The high concentrations that had to be applied orally, together with limited systemic resorption and efficacy against extra-intestinal parasites, indicate a rather non-specific mode of action of phenothiazine as a vermifuge. Interestingly, various reports imply that oxidized metabolites of phenothiazine, rather than the compound itself, could be responsible for its anthelmintic effect, and that these oxidized metabolites might interfere with the redox regulation or metabolism of susceptible parasites [1,40]. By 1952 it was shown that the oxidized phenothiazine metabolite phenothiazone was either lethal for flatworms like *Fasciola hepatica* (liver fluke) or that it at least had a paralyzing effect [41]. Oxidized metabolites of phenothiazine carrying a hydroxyl group generally possess two stable redox states, namely a reduced leuco-form (as in leuco-phenothiazone) and an oxidized quinoid-form (as in phenothiazone) – in a similar fashion to the situation in methylene blue (Fig. 2). These forms probably account for the observation that a variety of important dehydrogenases are inhibited by incubation with phenothiazine, potentially leading to a disruption of intracellular electron transport [1,40]. In the end, however, the relevant molecular mechanism of the anthelmintic effect of phenothiazine has never been clarified, which, to date, has prevented the design of improved and more-potent derivatives.

The taming of the shrew – how playing with the single reactive site of phenothiazine entailed an array of most precious drugs within just a few years

On the basis of Ehrlich's work with the putative antimalarial methylene blue, French researchers working with Paul Charpentier at Rhône-Poulenc Laboratories in Paris engaged in investigations of nitrogen-substituted derivatives of phenothiazine in the 1940s, one of them was the compound promethazine **5** (Phenergan, Figs. 3 and 4) [2,42]. Although no significant antimalarial efficacy was detected, these novel compounds were tested for other commercially interesting activities, among which happened to be antihistaminic effects. This translation was not too far-fetched, because it was Rhône-Poulenc that had just released phenbenzamine **4** (*N*-phenyl-*N*-benzyl-*N*′,*N*′-dimethylethylenediamine) in 1942 as the first fully synthetic antihistaminic [43], a structure onto which promethazine is rather nicely superimposable (Fig. 3).

In 1910, Sir Henry Hallett Dale, who became a Nobel laureate 26 years later, discovered histamine, termed β-iminazolylethylamine at that time, and evaluated its role in allergy [44]. Dale could show that in tissue preparations histamine induced diverse physiological responses including vasodilatation and hypotension, contraction of smooth muscles of bronchia and gut, and an increase in heart rate and contractibility. In animal studies [45], Charpentier's phenothiazine promethazine **5** turned out to be a more potent and specific antihistaminic than phenbenzamine **4** and, as such, it rather rapidly became a globally used drug for indications including allergy, eczema, pruritus, seasickness, emesis and asthma (Fig. 4).

Moreover, investigations by Henri Laborit, a French anesthesiologist, suggested that promethazine might be a useful co-medication to anesthetic drugs used in surgery [46]. And, indeed, under

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FIGURE 4

Phenergan (promethazine) is widely used as a sedating, antiallergic and antiemetic drug – as nicely visible from this packaging.

promethazine, pre- and post-operative nausea and emesis were reduced to a minimum [47]. More surprisingly though, promethazine also had a tranquilizing effect and reduced the stress of surgery and surgical trauma. Prerequisite for this unexpected result was the ability of promethazine, as a cationic – yet lipophilic – aromatic compound, to cross the blood–brain barrier (BBB) [48]. Having reached the CNS, promethazine primarily blocks histamine H_1-receptors, which are activated by afferents from the posterior hypothalamus, thus mediating wakefulness and attention [49]. Simultaneously, it was the later-discovered antagonism of dopamine receptors in the brain that avoided nausea and vomiting (Fig. 5). To date, promethazine has kept its status as one of the most useful antihistaminics despite the fact that the central, sedating effects are less desirable in most everyday applications (Fig. 4). This shortcoming of promethazine is resolved in more-recent H_1-antihistaminics such as cetirizine (Zyrtec®, 1990) simply by their BBB impermeability. Selective H_2-antagonists like ranitidine (Zantac®, 1981) or the BBB-permeable cimetidine (Tagamet®, 1976) are also relatively devoid of sedating effects; however, they can evoke other side-effects in the CNS [50]. Both compounds found wide application as inhibitors of stomach acid production in the 1980s.

Formulae containing promethazine and codeine are marketed as high-strength cough syrup in many countries, often over-the-counter, leading to their misuse for recreational purposes. In the southern USA, for instance, this mix has found appreciation in the Hip Hop and Rap scene when blended with soft drinks or candy ('Purple Drank' or simply 'Syrup') [51]. In overdoses, Purple Drank can lead to respiratory depression.

By the end of the 1940s another potent antihistaminic had come out of Rhône-Poulenc laboratories, chlorpromazine **6** (Largactil®). This compound, a close structural relative of promethazine **5** (Fig. 3), was too sedatory for daily use as antiallergic medication. Hence, Laborit used a combination of promethazine and chlorpromazine to initiate anesthesia and could, in consequence, employ much lower doses of conventional anesthetic drugs [46]. He noted that patients administered with chlorpromazine before surgery did not lose consciousness but, instead, became sleepy and lost anxiety [2,46], which reduced the stress for patients

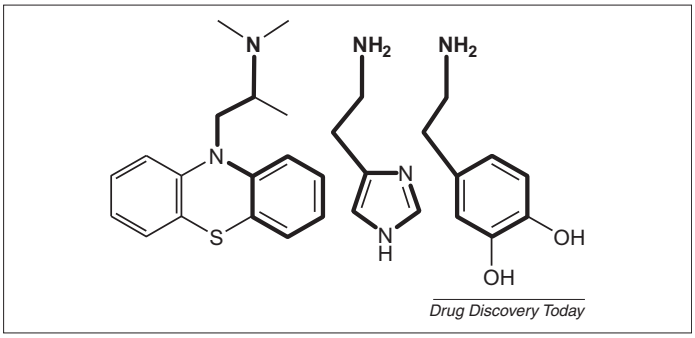

FIGURE 5

Structural similarities between promethazine, histamine and dopamine rationalize the pharmacological effects of promethazine and those phenothiazines that carry a (positively charged) substituent on the central nitrogen atom N^{10}.

and medical staff alike. The extraordinary efficacy of chlorpromazine as a sedative led to widespread exploration in clinical trials; still, it was not as suitable for anesthesia as promethazine because the activity lasted for too long, and it had a pronounced lowering effect on body temperature [52,53].

In consequence, chlorpromazine never found its 'home' in immunology nor in anesthesiology, but in a field that was to be changed fundamentally by this single compound – psychiatry.

The tempest – the introduction of chlorpromazine into psychiatry empties the wards of the lunatic asylums

Laborit had shared chlorpromazine **6** with psychiatric colleagues working in Paris because he speculated that the centrally sedating effects might be of value in the treatment of psychotic patients. In treating these patients with exceptional success [54], Jean Delay and Pierre Deniker found that chlorpromazine was not only a potent sedative but also literally an anxiolytic and antipsychotic drug [2,53–57], backed by animal studies demonstrating that chlorpromazine blocked certain forms of anxiety-related behaviour, for example the conditioned avoidance–escape response in mice [2]. Within a few years, the antischizophrenic efficacy of chlorpromazine led to a revolution in psychiatric thinking that is insufficiently described by the term 'practice-changing' (Fig. 6). For its immediately visible effects, chlorpromazine was globally administered to hospitalized psychiatric patients after 1952, well before controlled clinical trials [58–60] on different mental conditions including chronic overactive psychosis, agitated depression, insomnia, mania or melancholia were completed. In most of them, the treatment with chlorpromazine elicited a notable improvement.

Elkes and Elkes [56] give an illustrative description of the spectrum of effects of chlorpromazine in individual patients suffering from psychosis with overactivity. For example, a 69-year-old man who had spent 23 years of his adult life in the ward because of his recurrent manic attacks was found less overactive and noisy in his manic phases. He would not 'constantly run round the ward singing and shouting until he was hoarse' [56]. For the first time in 15 years he was able to leave the ward for a trip. Another patient, 62 years old, paraphrenic, was tremendously influenced by her auditory hallucinations. She loudly complained against an imaginary 'Mr. Knock', who would put 'filthy thoughts into her mind' [56].

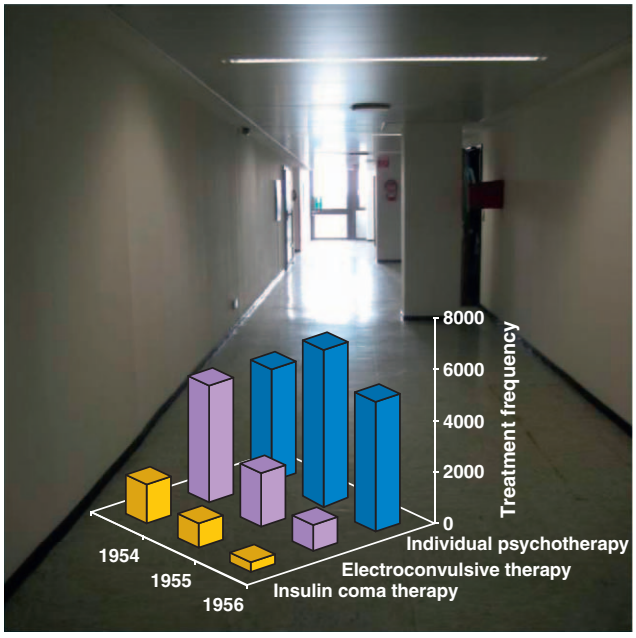

FIGURE 6

Chlorpromazine fundamentally changed practice in clinical psychiatry immediately after its marketing in 1953. Archaic and ineffective therapeutic concepts that had found frequent application before 1953, for example insulin coma therapy or electroconvulsive therapy, rapidly regressed, in spite of the universal trend of persistence in clinical medicine [118,119].

She showed aggressive–impulsive behaviour against staff and patients and had an obsession to scrub the floor all the time while shouting and talking. After chlorpromazine treatment for three weeks she 'did not bother any more with Mr. Knock', responded less to her hallucinations and became friendlier in general.

Before the advent of chlorpromazine the only medication strategy for patients with psychotic disorders was sedation. The idea of giving the central nervous system time to 'regulate' its imbalance by sleep or repose was widespread. The use of barbiturates, chloral or paraldehyde, was standard to control mental disorders with loud or aggressive behaviour [58–60]. Clinicians in the 1950s vividly described the disappearance of the foul-smelling odour from paraldehyde in the wards, together with the fact that the new drug enabled many of the hospitalized patients to 'function' in a way that they at least could participate in basic everyday life activities again. Because of the widespread use of chlorpromazine and related drugs in the 1950s, lunatic asylums became emptier all around the world [59] (Fig. 6), culminating in the statement that chlorpromazine was the drug that 'made modern psychiatry' [58].

Still, in the middle of the 1950s, it became clear that phenothiazine therapy was not yet free of side-effects, including weight gain, drowsiness and lethargy [56,57]. Most notably, though, it was observed that long-term treatment with chlorpromazine could entail a spurious form of motor impairment described as 'extrapyramidal motor disturbance' or 'extrapyramidal syndrome' (EPS). EPS comprises akathisia (motor uneasiness), tardive dyskinesia (involving involuntary movements of face or extremities) and parkinsonism (tremor, rigidity), which were eventually recognized

to be elicited by the dopamine receptor antagonist effects of chlorpromazine [61,62]. Unfortunately, it was quickly realized that dopamine receptor antagonism was also central to the antipsychotic action of these drugs, leading to the development of the dopamine hypothesis of schizophrenia [62,63]. For instance, Horn and Snyder had noted that antipsychotic phenothiazine derivatives mimicked the conformation of dopamine, and that chemical features putatively refining the said mimicry were associated with higher antipsychotic potency [64]. Via inhibition of dopamine receptors in the striatum, the typical Parkinson-like side-effects would thus be caused by the same molecular mechanism as the therapeutic effects of the phenothiazines [65,66]. Intriguingly, efforts by many distinguished pharmacologists resulted in a considerable portfolio of highly effective antipsychotic compounds in the 1950s, for example in phenothiazines such as perphenazine (1957), thioridazine **7** (1958) and perazine (1958), non-phenothiazines with close structural similarities, such as azaphenothiazines **10** (1959) or thioxanthenes **9** (1959), and completely unrelated compounds like haloperidol (1959), which all found their way onto the market but which also missed the goal of a clear, selective reduction of EPS side-effects.

Over the years, it has been established that chlorpromazine is an antagonist at diverse neurotransmitter receptors, not only the different dopamine receptor subtypes but also at histamine H_1, serotonin $5HT_{2A}$, adrenergic α_1 and muscarinic M_1 receptors [67]. At present, five dopamine receptor subtypes are known (D_1–D_5), and chlorpromazine has a non-selective affinity to all of them [68]. The inhibition of D_2-like receptors (D_2, D_3, and D_4) is thought to be primarily responsible for many of the antipsychotic effects, because more-potent phenothiazines usually exhibit lower K_D values for receptors of that family. For example, the affinity of chlorpromazine to the D_2 receptor is 5 nM, whereas the very potent compound fluphenazine **8** (Prolixin®, 1961) (Fig. 3) exhibits a K_D of 0.6 nM [67]. On the other hand, it has been recognized in the past two decades that dopamine dysregulation might not be the only explanation to the origin of psychotic disease [63,69], and there is certainly a whole spectrum of diseases summarized by the term 'psychotic disease' that differ in their molecular etiologies, opening the possibility that receptor selectivity tailoring could still yield optimized drugs in the future.

Hence, it was mainly the intention to suppress the EPS side-effects rather than to enhance drug potency on the so-called 'positive symptoms' of schizophrenia (hallucinations, delusions and disorganized speech and behaviour) that led psychiatrists and pharmacochemists to work continuously on chemically related structures, culminating in the development of clozapine in the early 1970s [2,70], a unique antipsychotic compound that is largely devoid of EPS side-effects, probably because of its relative sparing of D_2 receptors while not sparing the other members of the D_2 family, D_1 receptors or $5HT_{2A}$ receptors. Clozapine was, however, withdrawn from the market only four years after its introduction, for its potentially lethal side-effect agranulocytosis; but it was reintroduced in the late 1980s [71]. Despite the need of close hematological monitoring, this compound is essential for the treatment of patients refractory to other antipsychotic drugs [71,72]. In addition, clozapine, as well as certain other antipsychotics marketed since 1990, seemed to have a more visible impact on the so-called 'negative symptoms' of schizophrenia (apathy, affective flattening and emotional numbness). These drugs, among them risperidone (1995), olanzapine (1996) and aripripazole (2004), are frequently referred to as 'atypical' (or second generation) antipsychotics, because of their chemical structures ('typical' would be a 3-ring phenothiazine nucleus). To date, the atypical antipsychotics have largely taken over the antipsychotic market in industrialized countries, for their initially acclaimed activity on negative symptoms and lower incidence of EPS side-effects. In recent years, it has, however, become clear that the overall performance of these compounds compared with the phenothiazines or other first-generation drugs has been grossly overstated [73–76], especially because their benefit in terms of negative symptoms seems to be marginal at best [73]. Moreover, their global tolerability does not appear to be improved despite fewer EPS side-effects [74], and their primary efficacy could even be lower than that of first-generation drugs [77,78].

In conclusion, the use of phenothiazines to treat psychosis and schizophrenia is clearly not as exclusive and extensive as it was between the 1950s and 1990s. Nevertheless, they still constitute an indispensable tool in the pharmacology arsenal, illustrated by the fact that two out of three antipsychotic drugs ranked in the WHO List of Essential Medicines are phenothiazines, namely chlorpromazine **6** and fluphenazine **8** [3]. For their proven efficacy, tolerability and cost-effectiveness [75–78], these compounds were and remain drugs of truly global value.

The winter's tale – the unique redox activities of the phenothiazines and their translation from the engineering world into biomedicine

Unnoticed by the physicians prescribing phenothiazine as an anthelmintic and using it as a toolkit for the synthesis of an ever increasing number of biogenic amine receptor modulators, phenothiazine had already begun its second 'career' in the engineering world. Chemists and engineers noted the exceptional ease with which phenothiazine **3** served as a hydrogen donor in free-radical chain-reactions, thereby acting as a chain-breaking antioxidant. By the end of the 1940s Charles Murphy *et al.* had demonstrated that phenothiazine was highly efficient in preventing oxidation of turbo-jet-engine lubricants even at temperatures beyond 100°C [4]. In 1952, he patented the use of 0.2% phenothiazine as a preservative, additive and oxidation-inhibitor for synthetic engine oils [79]. Besides, he was one of the first to characterize the capacity of phenothiazine to exist as a resonance-stabilized free-radical under various conditions (Fig. 8), thereby accounting for its antioxidative activities, an interpretation that was extensively confirmed later [80–82]. Numerous derivatives of phenothiazine for similar applications were synthesized and patented in the 1950s and 1960s [82,83], with ongoing activity to date.

The versatile redox properties of the phenothiazines have recently also sparked investigations in various other technical fields in which redox control is pivotal. To name just a few of these, the prevention of overcharges in lithium ion batteries [84], the construction of novel devices for solar energy storage [85] and the photo-control of chemical reactions [80] have all been investigated. The common property of these technical phenothiazines, in contrast to all histamine- or dopamine-receptor binding phenothiazines, was the presence of a free, unsubstituted nitrogen atom that had been dissected as the indispensable carrier of their

redox activity [4,81], an essential that was reproduced several decades later in a totally different application, namely when phenothiazines were characterized as chemicals that potently protected cells, especially neurons, from oxidative cell death [81,86]. Phenothiazine, an antiseptic and anthelmintic, mother compound to numerous antiallergic and antipsychotic drugs, thus became assigned just another and totally novel label because the importance of its formerly technical redox properties for biological and medical questions was realized. Seminal in this development was the recognition that uncontrolled oxidative processes involving free radicals were pivotal to diverse pathological processes, ranging from autoimmune disease to neurodegeneration and aging [87–92]. This fact led to the widespread acceptance of the term 'oxidative stress' in the early 1980s [87], as briefly discussed in the following.

Oxidative stress, originating either from an increased production of intracellular oxidants or from an impaired defence against normal production rates, implies a permanent challenge for respiring cells. These oxidants, primarily reactive oxygen species (ROS), are mainly generated in the mitochondria as by-products of respiration carried out by the electron transport chain. For example, superoxide anion radicals ($O_2^{\bullet -}$) are generated by complex I from oxygen and reduced flavin moieties ($FMNH_2$). The generated superoxide is detoxified by superoxide dismutases (SODs), which catalyze the reaction of two superoxide radicals to hydrogen peroxide (H_2O_2) and oxygen. Hydrogen peroxide is also produced by numerous oxidases, for example in the peroxisomes. Peroxides are particularly relevant ROS because of their interaction with redox-active metal ions, such as iron (Fe) or copper (Cu), which facilitate the formation of primary free radicals from these peroxides [87,88], most notably the highly reactive hydroxyl radical ($^{\bullet}OH$). Each cell possesses various indispensable enzymatic and non-enzymatic mechanisms providing a relatively high tolerance of eukaryotic cells to ROS. Under pathologic conditions, however, relevant bricks from this wall can be lost or become dysfunctional. Regarding neurodegenerative diseases, almost all of these debilitating disorders have been linked, whether causally or as a downstream phenomenon in the progression of the disease, to oxidative stress [88–90]. For example, oxidative stress induces neuronal lipid peroxidation, thereby impairing calcium homeostasis and exacerbating glutamate-triggered excitotoxicity [89,90]. Moreover, membrane fluidity is altered by an increased content of lipid hydroperoxides, which probably affects the appropriate response of multiple types of neurotransmitter receptors to their ligands.

In the end, oxidative stress might induce cell death displaying either features of apoptosis or necrosis to varying degrees [89,91], even if it is known that authentic apoptosis is exceedingly rare in the CNS and does not occur in chronic progressive neurodegenerative disease [93]. In chronic diseases, such as Alzheimer's or Parkinson's, there is a selective and highly characteristic loss of neurons (or neuronal substructures) in particular areas of the brain that are often connected to a similarly specific, preceding accumulation of ROS [90,94,95]. In more acute neurodegenerative syndromes, such as cerebral ischemia or trauma, increased levels of oxidative damage have likewise been demonstrated. As one outcome of these findings, which were mostly published during the 1980s and 1990s, antioxidants have entered stage in medicine and pharmacology, even if a causal therapy of a major neurodegenerative disease (by means of an antioxidant strategy) has not been achieved so far [94,95].

All's well that ends well – on the rise of methylene blue and other phenothiazines as experimental agents to treat neurodegenerative disease

Tying in on earlier work on technical lipid peroxidation [96], Melvin Yu *et al*. from Eli Lilly laboratories reported, in 1992, that *N*-unsubstituted phenothiazine compounds were potent lipid peroxidation inhibitors in isolated brain lipids, and cytoprotective agents in neuronal cell culture, and they explained this property with the low oxidation potential and the high lipophilicity of these compounds [86]. In an *in vivo* approach of global and focal cerebral ischemia in rats, Yu *et al*. demonstrated a reduction of post-ischemic damage by pretreatment with 2-(10*H*-phenothiazin-2-yloxy)-*N*,*N*-dimethylethanamine hydrochloride, a cationic phenothiazine derivative [97]. Interestingly, from several novel phenothiazines that had been synthesized, none was significantly more potent than the parent compound phenothiazine itself [86]. Hence, in their subsequent *in vivo* study, they chose to investigate a

TABLE 1

Protective EC_{50} values of phenothiazine, and of several standard antioxidants in biochemical, cellular and *in vivo* models of neurodegenerative disease

	Cell survival				Oxidation	Efficacy *in vivo*	
	HT22 cells/ glutamate	SK-N-MC cells/H_2O_2	SH-SY5Y cells/MPP^+	Midbrain neurons/MPP^+	Pig brain membranes/ ascorbate	*C. elegans*/MPP^+	*C. elegans*/ rotenone
***N*-Acetylcysteine**	70 μM	600 μM					
Trolox C	40 μM	20 μM					
17β-Estradiol	13 μM	10 μM			10 μM		
Resveratrol	10 μM	5 μM					
Quercetin	6 μM	4 μM					
Iminostilbene	0.07 μM	0.08 μM	0.02 μM	0.02 μM	0.2 μM	0.08 μM	0.08 μM
Phenothiazine	0.02 μM	0.02 μM	0.02 μM	0.04 μM	0.06 μM	0.06 μM	0.05 μM

Cell survival following a cytotoxic dose of the indicated prooxidative toxins (glutamate or H_2O_2 or MPP^+) was quantified. Lipid peroxidation was measured in pig brain membranes challenged with pro-oxidative concentrations of ascorbate. *Caenorhabditis elegans* nematodes were intoxicated with mitochondrial complex I inhibitors leading to selective pro-oxidative death of dopaminergic neurons *in vivo* (MPP^+ is 1-methyl-4-phenylpyridinium). The indicated concentrations of the listed antioxidants led to 50% protection from the effect of the applied toxins. HT22 are murine clonal hippocampal cells, SK-N-MC and SH-SY5Y are human neuroblastoma cells. Data are from Refs. [98,102,103,121].

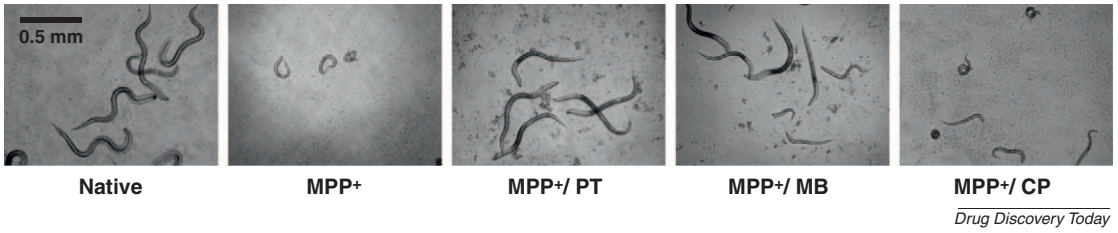

FIGURE 7

1-Methyl-4-phenylpyridinium (MPP^+) is a respiratory chain complex I inhibitor inducing dopaminergic neurodegeneration in a wide range of species from invertebrates to humans. MPP^+ treatment constitutes one of the classic animal models of Parkinson's disease. Its toxicity is thought to be largely dependent on pro-oxidative effects evoked by its complex I inhibitory activity. In larval *Caenorhabditis elegans* nematodes MPP^+ leads to readily visible larval arrest and death of the animals. Sub-micromolar concentrations of phenothiazine (PT) and, somewhat less effectively, methylene blue (MB), but not of chlorpromazine (CP), protect *C. elegans* from dopaminergic neurodegeneration, which is evidenced by their normal growth and development into fully fertile adults.

more soluble and thus more easily applicable cationic derivative of phenothiazine, despite the fact that it had been only half as potent *in vitro* as phenothiazine itself. Unfortunately, Yu *et al.* also noted toxicity at the higher of two doses they employed in a rat model of permanent middle cerebral artery occlusion (20 mg/kg/h for 24 h). Considering that, from a chemical point of view, the administered compound was a cationic detergent, the latter result might not have been totally unexpected.

These pioneering studies were not followed up until, in 2000, the unique potency of the plain parent structure, phenothiazine itself, was again demonstrated and also shown to comprise protection from glutathione depletion and in various other cell culture paradigms of oxidative neurodegeneration [81]. It was shown that the decisive redox activity to provide neuroprotection was shared by phenoxazine and other bis-arylimines such as 5*H*-dibenz[*b*,*f*]azepine (iminostilbene), and a quantitative SAR model based on computational chemistry was reported. Instead of a low oxidation potential (a property shared also by many non-antioxidants) the computational chemistry model identified more relevant variables to predict cytoprotective properties in aromatic imines – that is the difference in heat of formation during radicalization, the energy of the lowest unoccupied molecular orbital (LUMO) of the corresponding radical and, somewhat less importantly, the octanol–water partition coefficient (log *P*, a value for lipophilicity) [81]. By these investigations, it became clear that aromatic imines are, in general and by virtue of their structural and quantum chemical build-up, much more potent chain-breaking antioxidants than phenols, whose radicalized forms are intrinsically less stabilized by mesomerism than bis-arylimines such as phenothiazine. The rather weak antioxidant effects of phenols in living cells have frequently been noted [81,98–100]; nevertheless, investigations into phenols have dominated the field of antioxidant neuroprotection until recently, perhaps because natural phenolic compounds such as flavonoids and stilbenes occurring in green tea or red wine were just too attractive to be abandoned [99].

More lately, the protective action of phenothiazine and its direct antioxidant mechanism have both been confirmed in mitochondrial toxin-based models of Parkinson's disease. In particular, the sporadic form of this neurodegenerative disorder of the dopaminergic system seems to be caused by a combination of increased ROS production and mitochondrial dysfunction [101]. To protect different types of dopaminergic cells, remarkably low concentrations of phenothiazine of approximately 20–60 nM were sufficient (Table 1) [102]. In an investigation on toxin-treated, transgenic *Caenorhabditis elegans* worms modelling Parkinson's disease, the high potency of the phenothiazines and the structural prerequisite of a free, unsubstituted *NH* bond [81] was confirmed and shown to be independent of any dopamine agonist or antagonist effects (Fig. 7). Hence, chlorpromazine **6** and all other *N*-substituted phenothiazines were ineffective [103], whereas methylene blue **1** showed a notable degree of protection (Figs 7 and 8). Intriguingly, neuroprotection by methylene blue **1** has recently been established in a variety of mammalian models of neurodegenera-

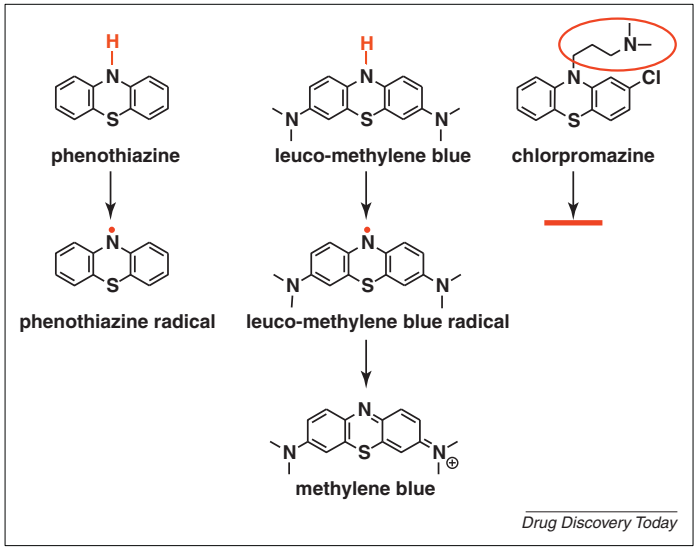

FIGURE 8

Radical chemistry of three prototypical phenothiazines. Phenothiazine and leuco-methylene blue can both undergo radicalization by homolytic hydrogen abstraction. The resulting phenothiazinyl radicals are exceptionally well stabilized by mesomerism (electron delocalization), making phenothiazine and leuco-methylene blue very good chain-breaking antioxidants. Substituted phenothiazines, such as chlorpromazine and all other marketed antipsychotics, cannot undergo fast radicalization by hydrogen abstraction; they thus lack antioxidant activities in biological settings. A speciality of the leuco-methylene blue radical is its potential to further donate a single electron, leading to the stable methylene blue cation. As a two-electron donor, leuco-methylene blue can therefore catalyze a process called redox cycling. The biological value of redox cycling is strongly dependent on the precise situation; it is usually discussed as a pro-oxidative reaction, for example as the origin of dopamine neurotoxicity [120]. However, it can also bring about antioxidant effects if it prevents the formation of superoxide in the electron transport chain (compare Fig. 9) [108].

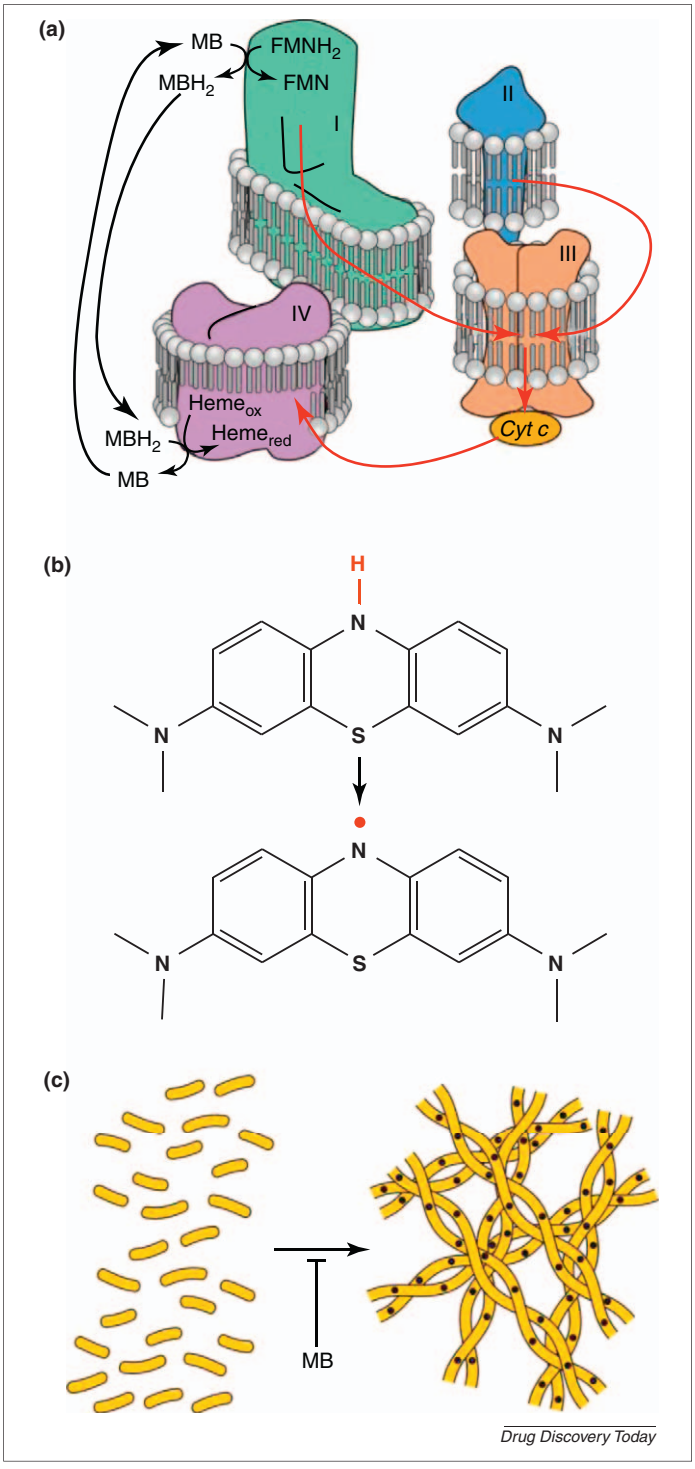

FIGURE 9

Three possible mechanisms of how methylene blue might interfere with the pathogenetic processes in Alzheimer's disease. **(a)** Stabilization of mitochondria via redox cycling. According to this mechanism, methylene blue would act as a metabolic antioxidant to reoxidize complex I in case of 'electron congestion', which is associated with high superoxide production. Reoxidation of the resulting leuco-methylene blue could occur at complex IV or on other hemoproteins such as cytochrome c. Normal electron flux is indicated by red arrows. Black arrows trace the postulated diffusional path of methylene blue. **(b)** Protection from oxidative stress by direct radical scavenging. In this model, administered methylene blue would be enzymatically reduced to leuco-methylene blue, which would then act as a direct antioxidant (a free radical scavenger) in all compartments of the cell, including extra-mitochondrial sites. If this scenario was correct, phenothiazine itself should be as effective as methylene blue. **(c)** Inhibition of tau aggregation. Here, methylene blue would prevent the formation of tau aggregates termed 'paired helical filaments' (PHFs). Together with amyloid plaques, PHFs are one of the histological hallmarks of Alzheimer's disease, and they might be causative to the disease according to one of the prominent theories. If this concept was correct, other inhibitors of tau aggregation should be as effective as methylene blue.

tion, for example, in two rat models of striatal neurotoxicity [104,105] and in mouse and rat models of retinal degeneration and optic neuropathy [106,107].

Looking at the structure of methylene blue **1** (Fig. 8), the absence of a homolytically cleavable *NH* bond would clearly predict rather weak, if not absent, activity as an antioxidant [81]. *In vivo*, however, provided sufficient metabolic reducing equivalents, methylene blue **1** is readily reduced to its leuco-form **2**, for example, by mitochondrial NADH dehydrogenase [108] or other, mostly NADPH-dependent, flavoenzymes (Figs 2, 8 and 9). Leuco-methylene blue **2**, in turn, is predicted to be a direct antioxidant of a similar potency to phenothiazine **3**. Moreover, as a lipophilic cation, it accumulates in mitochondria, at least as long as they maintain their membrane potential. Furthermore, Atamna *et al.* have reported that methylene blue retards the replicative senescence of primary human fibroblasts [108] by blocking the externally measurable mitochondrial oxidant production, and a protective redox-cycling of methylene blue between respiratory chain complex I and IV was suggested as the molecular mechanism (Fig. 9a). Hence, although it might be attractive to speculate that in many of the investigated models of neurodegeneration, it is leuco-methylene blue that is the true carrier of protective activity, and that plain phenothiazine should basically do the same (except being blue), it is equally possible that two-electron redox-cycling processes unique to methylene blue are involved, in which case plain phenothiazine should be lacking any effect (Fig. 8). Whatever its precise mechanism, methylene blue certainly constitutes a tantalizing drug candidate for degenerative diseases in which mitochondrial dysfunction is causally involved.

This conclusion is underpinned by the fact that methylene blue has shown clinically beneficial effects in a multitude of other conditions, which demonstrates its safety and applicability. For instance, methylene blue **1** is effective against methemoglobinemia, because of its facile intracellular reduction to leuco-methylene blue **2**, which then reduces methemoglobin (Fig. 2). An accumulation of >1% methemoglobin (iron-oxidized hemoglobin), whether caused by an inherited metabolic defect or caused by toxins, leads to a bluish colouration of the lips, nails and skin. In the 1960s the treatment of a cluster of families suffering from an inherited deficiency of NADH-cytochrome B5 reductase (methemoglobin reductase), who thus displayed a chronic blue coloration from methemoglobinemia, was reported in the media and made methylene blue rather famous for its seemingly paradoxical activity: methylene blue, at a dose insufficient to stain the body blue by itself, reduces methemoglobin and thus abolishes the human stain; hence, the 'blue people of Troublesome Creek, Kentucky', turned 'pink' again [109].

Administered to vasoplegic (hypotonic, vascularly paralyzed) patients after cardiac surgery at reasonable doses (1.5 mg/kg), methylene blue significantly reduced mortality [110]. Given to ifosfamid-treated cancer patients at several 100 mg/d, a significant

reduction in drug-induced neurotoxicity was reported [111]. In both cases, redox-dependent mechanisms of inhibition of flavin-carrying enzymes (nitric oxide synthase and monoamine oxidase, respectively) could have been involved. Following intracavernosic injection, methylene blue has also been applied in pharmacologically induced priapism (persistent, painful erection) [112]. As the therapeutic mechanism here, the inhibition of guanylate cyclase to decrease cGMP production has been cited. After all, methylene blue has also returned to its home base in the past decade, as probably the most enduring drug candidate ever, to be investigated as a putative adjuvant for the treatment of malaria.

Yet, overshadowing all other phenothiazine drug development news in the past decade, results of an apparently successful clinical study of methylene blue in Alzheimer's disease were presented at the International Conference on Alzheimer's Disease (ICAD) held in July 2008 in Chicago.

Various hypotheses exist on the cause of Alzheimer's disease, among them the still prevailing amyloid β protein (Aβ) hypothesis, according to which the aberrant processing of an integral membrane protein (APP) leads to the accumulation of a ~40 amino acid peptide, whose stepwise extracellular aggregation to Aβ plaques would gradually cause synaptic and neuronal loss. As one of the toxic effector mechanisms, oxidative stress has been cited widely, which would open a clear possibility for antioxidant treatment [88,90,98]. A second, somewhat less frequently cited, hypothesis assigns particular pathological relevance to the intracellular aggregation as paired helical filaments (PHFs) of a microtubule-associated protein (tau protein) in an abnormally phosphorylated form. Clearly, not all aspects of these hypotheses are mutually exclusive.

Returning to methylene blue, findings presented by Claude Wischik at ICAD indicated an unprecedented strong beneficial effect of 180 mg/d methylene blue in moderate Alzheimer's disease [113]. Moreover, by 1996, researchers with Wischik had published data indicating that tau aggregation *in vitro* could be inhibited by methylene blue (IC_{50} 3.4 μM). Concomitantly, methylene blue derivatives with ~30× higher potency were described, among them the desmethyl derivatives azure A and B [114]. Hence, it was claimed (by third party) that, by showing efficacy of methylene blue in Alzheimer's disease, the predominating pathomechanism would have been clarified. However, even if the results with methylene blue were confirmed in additional clinical studies, the latter conclusion would be premature, given the substantial number of mechanisms by which methylene blue acts *in vivo*. Three of the possibly most plausible candidate mechanisms in Alzheimer's disease are depicted in Fig. 9, namely stabilization of mitochondria via redox cycling [107,108], protection from oxidative stress by direct radical scavenging [81,103] and inhibition of tau aggregation [114], yet others could have also been mentioned on a more speculative basis (e.g. the inhibition of nitric oxide production).

Adding further complexity, the inhibition of aggregation by lipophilic cations such as methylene blue seems to be a rather promiscuous affair. In 2007 methylene blue was tested for its effects on Aβ aggregation *in vitro* and it was found to reduce oligomerization, but to increase fibrillization, a pattern that could, according to current thinking, be beneficial [115]. Concentrations of ~30 μM were necessary to elicit these effects. Moreover, various phenothiazines even prevent protein misfolding as in the case of the prion protein [116]. Prion proteins are ubiquitous cell-surface proteins that cause prion diseases such as Creutzfeldt-Jakob when they, by some trigger, start changing their conformation and misfold. In this model, only *N*-substituted, cationic phenothiazines such as chlorpromazine **6** were effective at ~10 μM concentration, whereas phenothiazine **3** itself had no effect. The unique binding properties of the cationic phenothiazines have also been investigated in models of infectious disease, related here to RNA binding [117].

Beyond any doubt, the results presented by Wischik *et al.* at ICAD have led to a reinforced interest in the exceedingly versatile lead structure phenothiazine. And, given the fact that phenothiazine and leuco-methylene blue are nanomolar potency chain-breaking antioxidants in the lipid bilayer, the game is still open whether *in vivo* effects seen in Alzheimer's disease (if confirmed) are finally attributable to aggregation inhibitory properties (with EC_{50} values of 3–30 μM), antioxidant activities (with EC_{50} values of 10–100 nM) or mechanisms yet to be discovered. Serendipitously enough, methylene blue might thus become a chief judge on the battle of the true origin of Alzheimer's disease, a role that probably even Paul Ehrlich would not have deemed possible for his favourite histochemical dye.

Acknowledgements

The authors would like to thank J. Mocko for providing images of phenothiazine-treated *C. elegans*, M. Plenikowski for generating graphical illustrations on the actions of methylene blue, and M. Schreckenberger and C. Behl for their support. The authors' experimental work on phenothiazines is supported by the Neuro Graduate School and the Interdisciplinary Research Centre for Neurosciences of the University of Mainz.

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