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Review

Dyes in the development of drugs and pharmaceuticals

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Abstract

Research during the early years of dye synthesis produced compounds such as methylene blue and acriflavine which were used as biological stains. The selectivity of such compounds for ''non-economic'' cells such as pathogenic bacteria or tumour cells gave rise both to the principle of selective toxicity, and to the development of modern drugs, for example in the field of tropical medicine.

The use of dyes in therapy is again gaining credence today, given the efficacy of light-activated drugs based on dye molecules against drug-resistant organisms such as MRSA. In addition, older drugs developed from dye chromophores may again be of use in the clinic due to the continuing rise of the ''Superbugs''.

Keywords: Dyes; Disease; Drug development; Pharmaceuticals; Drug resistance

1. Introduction

Dyes and drugs. Both part of the chemical industry, but one might be excused for thinking that they were at opposite ends of the spectrum. Certainly traditional chemical synthesis in the two areas is quite different, as are ideas concerning product purity. However, the two parts are closely linked, much more so than is popularly believed, and the links cannot lie too far in the past, since the chemical industry is not much more than 150 years old. In fact, the pharmaceutical industry grew out of the dye industry just before World War II.

In medical histories, penicillin is cited *ad nauseam* as a wonder drug, but the fact remains that it was discovered by accident rather than by design, and was preceded into the clinic by drugs such as the sulfonamides, which came about as part of an organised drug development programme based on dyes (see below). In today's hospitals penicillins, along with many other drugs from the so-called ''antibiotic era'', do not enjoy such an exalted position with infectious disease specialists due to rampant drug resistance.

2. From laboratory to clinic: 19th century pioneers

The use of extracts of plants to mark or stain the skin (e.g. woad, henna) pre-dates the work of Christian Gram by several millennia. However, Gram's use of synthetic dyes to differentiate between bacterial and other cells was of much greater utility to mankind. The Gram method (still) uses a combination of crystal violet (gentian violet), safranine and iodine to stain bacteria. This allows differentiation between bacterial and human cells, for example, but depending on the coloration of the bacteria these are classified as *Gram-positive* (if purple/blue), or *Gram-negative* (if red-brown). The difference in staining is due to a gross difference in cell wall structure. Thus, we can easily differentiate between infamous pathogenic bacteria such as the hospital ''Superbug'' methicillin-resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli* O157 which has been implicated in several serious food-poisoning outbreaks – the first bacterium is denoted Gram-positive, the second Gram-negative.

Other scientists took Gram's work further: Robert Koch realised that the Gram stain did not work well on the bacteria responsible for tuberculosis (*Mycobacterium tuberculosis*) and came up with a staining procedure which required removal

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of the dye by acid. Tuberculosis germs are now known as *acid-fast* bacteria.

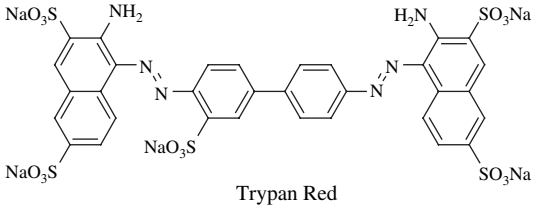
Perhaps the biggest breakthrough was made by Paul Ehrlich in the late 19th century. Ehrlich realised that there was a *selectivity* being demonstrated by certain dyes used as microbial stains, and, as a chemist, tried to alter the dyes structurally to increase their specific concentration by the microbes sufficiently to have a toxic effect. Ehrlich found that it was possible to demonstrate this, in terms of therapeutic potential, with small infected animals and eventually reported the cure of two human patients with a microbial disease – malaria – in 1891 [1]. This was the first documented cure of microbial disease with a synthetic chemical. It is ironic that Perkin's mauve resulted from his attempts at synthesising the antimalarial drug quinine from aniline, while Ehrlich used the aniline dye methylene blue as an antimalarial drug. Unsurprisingly, methylene blue and its derivatives are excellent stains for malarial parasites (*Plasmodium* spp.), and the standard stains (Romanovsky, Giemsa) used today are based on methylene blue or azure dyes [2]. Ehrlich derived the terms *selective toxicity* and – more importantly – *chemotherapy* from his work on dyes and stains.

3. Tropical medicine

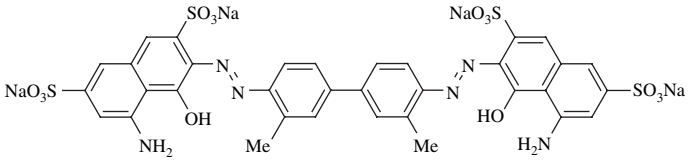
At the beginning of the 20th century, Germany had considerable overseas colonies, particularly in Africa. This meant that one of the major drivers of Ehrlich's work was tropical disease. As with malaria, he made significant discoveries concerning the chemotherapy of human African trypanosomiasis (HAT), a parasitic disease transmitted by the bite of the tsetse fly and often manifested as neurological damage and ''sleeping sickness''. Again using dyes and the selective staining paradigm, Ehrlich showed that the disease could be arrested in a mouse model, particularly by azo dyes such as those now known as Trypan red and Trypan blue (Fig. 1). Unfortunately, these were not successful in HAT.

Wilhelm Roehl and Carl Browning were both students of Ehrlich and both carried on his work through their own researches. Roehl worked for the German chemical company Bayer on, among other things, HAT. The shortcomings of the azo dyes coupled with the clinical disadvantage of tissue staining led to the replacement of the azo (−N=N−) linkages with urea (−NH−CO−NH−) and carboxamide (−CO−NH−) groups. In turn, this change gave rise to a successful, non-staining compound, originally called Germanin, but now known as Suramin (Fig. 1). This is still a front line treatment for HAT [3].

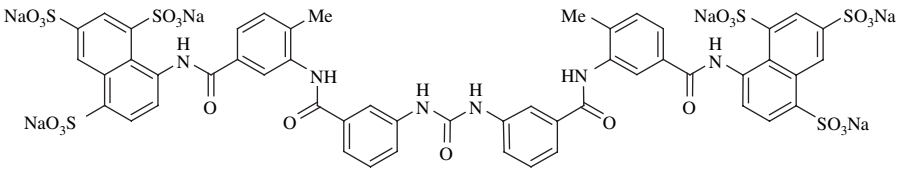
Browning followed up Ehrlich's work on the acridine dye acriflavine, both for trypanosomiasis and, later, as an antibacterial (see below). As with Trypan red and Trypan blue, Ehrlich showed that acriflavine was trypanocidal in mice but not in humans [4]. Consequently, Browning and his co-workers investigated the effects, not only of related acridines, but also of isomers and partial forms of the acridine molecule. From this work, the efficacy of the phenanthridinium class (Fig. 2) became apparent, and drugs based on this system were introduced, such



Trypan Red



Trypan Blue



Suramin (Germanin)

Fig. 1. Suramin and the *Trypan* azo dyes.

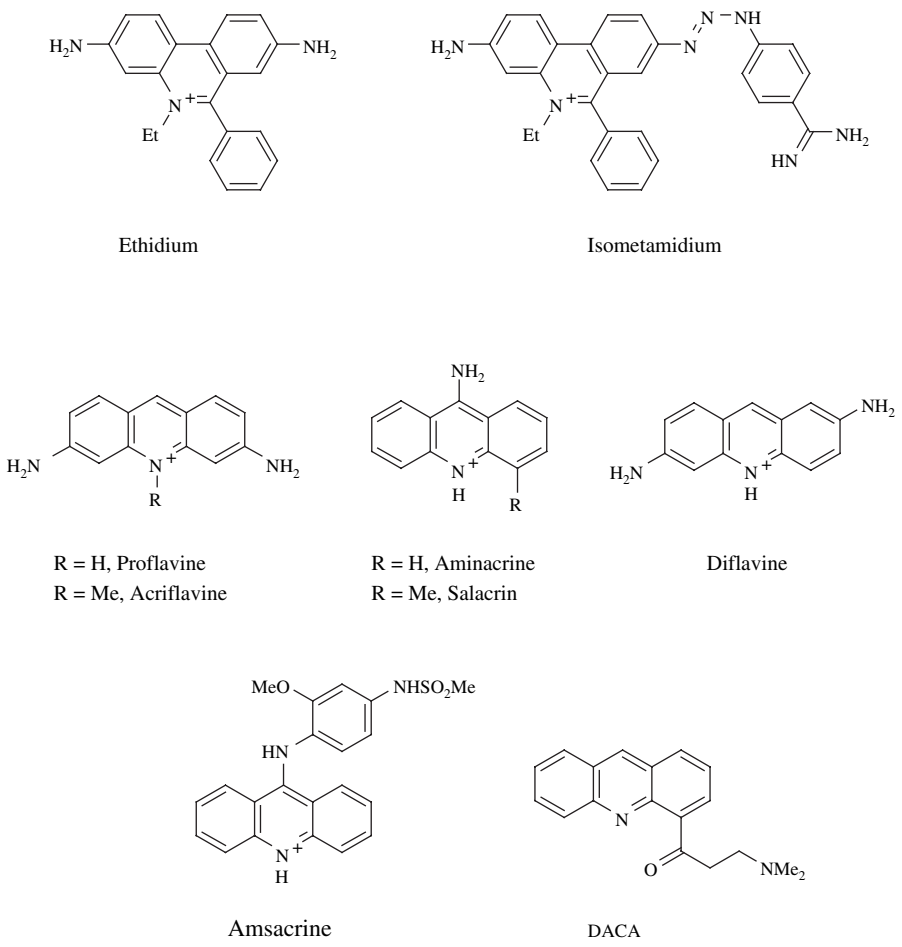


Fig. 2. Phenanthridine and acridine-derived drugs.

as ethidium bromide (Fig. 2). Although there were concerns about the deleterious side effects of this drug, other related phenanthridinium derivatives have replaced it, especially in veterinary medicine. Samorin (isometamidium, Fig. 2) currently enjoys widespread use in sub-Saharan Africa [5].

Due to Ehrlich's original use of methylene blue as an antimalarial, this swiftly became a lead compound in future drug research. Roehl and his co-workers at Bayer and later at IG Farben developed new derivatives via side chain variation (Fig. 3). Although improvements were made relative to the activity of methylene blue, the resulting compounds were still – unsurprisingly – an intense blue, which was considered a disadvantage in terms of patient compliance [6]. Consequently, other chromophores were used with the new aminoalkylamino side chains, resulting in several successful antimalarial drugs, such as chloroquine (based on the quinoline chromophore) and mepacrine, a bright yellow acridine derivative (Fig. 3). When methylene blue was shown to have a beneficial effect in mania, the side chain/chromophore variation process was used again. Such was the success of the combination of different chromophores that the new side chains can be seen in many different modern drugs, such as antihistamines, antipsychotics and antidepressants (Fig. 4). Two of the most highly used drugs in history, chloroquine and chlorpromazine (Thorazine) can thus be traced directly to methylene blue.

Unfortunately, drug resistance has become a considerable problem in the treatment of malaria. Chloroquine resistance is now widespread and several other drugs have been introduced. Fittingly perhaps, clinical trials using methylene blue were recently commenced (2004) in Burkina Faso [7].

4. Disinfection

As well as his discoveries involving the phenanthridine-based trypanocides, Carl Browning was involved in antisepsis research from early in his career. Again following Ehrlich's lead, he investigated the use of acridine dyes as antibacterial agents. His work was aided by the exigencies of World War I and the dreadful loss of life in the trenches, much of which was directly caused by wound infection. Browning and his collaborators introduced "dye therapy", employing the acridine derivatives proflavine and acriflavine (Fig. 2), to treat battle wounds in base hospitals to significant effect in comparison to the contemporary antiseptics.

Browning's work on antibacterials continued in the 1920s and 1930s, on a similar basis to his trypanocidal endeavours, and included a clinical trial of a cyanine dye based on the quinoline nucleus [8]. When large scale wound therapy was required again during World War II, the aminoacridines were again to the fore, along with the sulphonamide drugs. Adrien

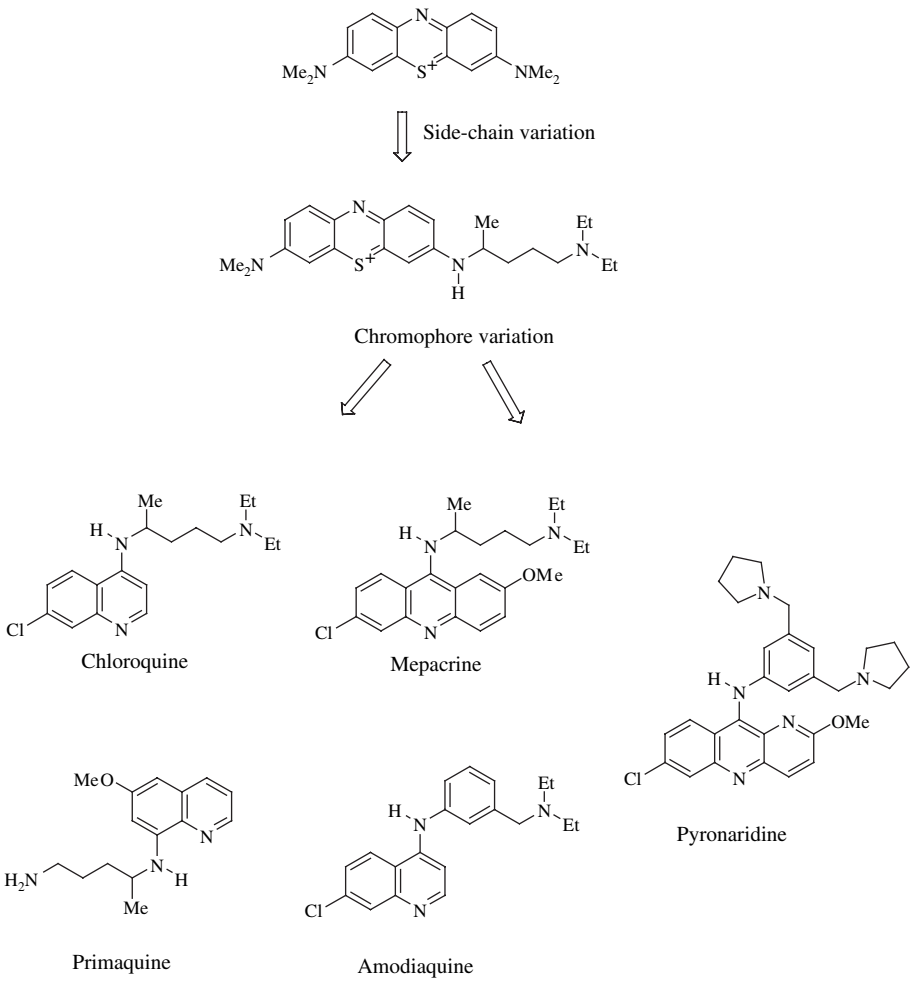


Fig. 3. Pyronaridine and antimalarial drugs derived from methylene blue.

Albert, in turn, carried on the research into acridine-based drugs, introducing the acridine derivatives Aminacrine and Salacrin, based on 9-aminoacridine, and Diflavine, (2,6-diaminoacridine, Fig. 2) [9]. Interestingly, the combination of aminoacridines and sulfonamides also proved highly effective in wound disinfection [10].

Albert's contribution to acridine chemistry remains immense. He was responsible for the use of aminoacridines in wounds and also for allied use of the acridine antimalarial compound Mepacrine (Fig. 3). His initial rationale for the new derivatives used in wound therapy was that they are non-staining (earlier derivatives had stained the skin yellow), and this may have been the reason for the infrequent use of Diflavine which, although a highly effective antibacterial, stains the skin red [11]. Albert also established the structure–activity relationships necessary for effective antibacterial activity in the aminoacridines – cationic nature in conjunction with a continuous planar molecular area equivalent to at least three benzene rings. It is now established that aminoacridines such as proflavine act on bacterial DNA, altering its structure via intercalation between the base pairs, thus inhibiting replication [12]. The anti-nucleic acid effects of acridines are now well accepted and there are various anticancer drugs based on this, such as amsacrine and DACA (Fig. 2) [13,14], although, paradoxically, this has made it difficult to progress new active acridines into the clinic due to fears over possible effects on human DNA. Proflavine remains in use today as a topical antibacterial in burns units, but other examples were made obsolete by the much safer β-lactam agents, i.e. penicillins etc.

The DNA-mediated antibacterial activity of the aminoacridines has also led to their use as antiviral agents, targeted at viral nucleic acid. Recent use has included the successful treatment of AIDS patients with acriflavine [15].

5. Azo dyes and Sulfa drugs

One of the most famous instances of dye use in therapy must surely be that of Prontosil, an azo dye, for which Gerhard Domagk of Bayer/IG Farben won the Nobel Prize for medicine in 1939. Domagk, like Ehrlich, Roehl and Browning before him, tested series of dyes as potential antibacterial agents, discovering the significant activity of Prontosil (sulfamidochrysoidine, Fig. 5) in animals in 1932 [16]. Within a few years, of course, it was discovered that the active agent was not the azo dye, but one of its reduction products, sulphanilamide (Fig. 5), Prontosil thus behaving as a pro-drug. *Prontosil rubrum* was widely used, and shown to be highly effective in the treatment of serious bacterial illness, such as septicaemia

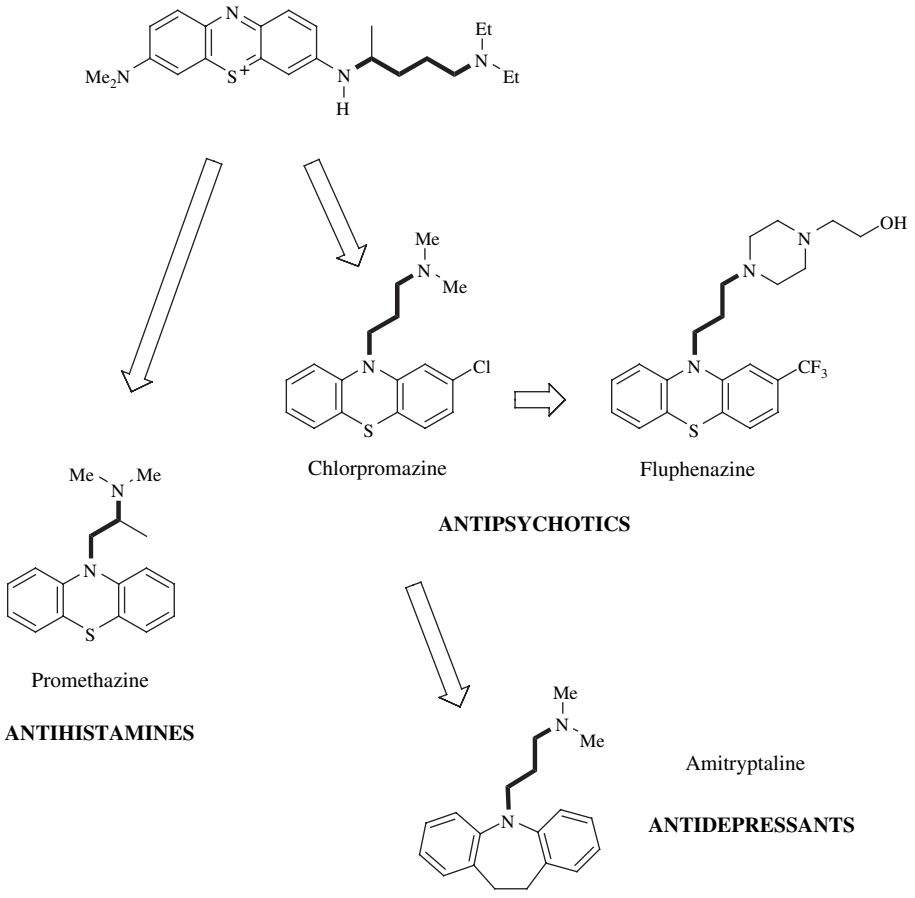


Fig. 4. Other drugs derived from methylene blue.

and puerperal (childbirth) fever [17]. However, once the active agent had been recognised, sulphonamide derivatives were rapidly patented and released to the clinic, beginning what many accept as the ''Golden Age of antibiotics'', some 4–5 years before the widespread introduction of the penicillins, their derivatives, other wonder drugs and, regretfully, ubiquitous multidrug-resistant bacteria.

In today's hospitals single sulphonamide drugs are used sparingly, mainly due to long-established sulphonamide resistance. However, the combination of sulfamethoxazole and trimethoprim (Bactrim, Fig. 5) remains an everyday treatment for bacterial infection of the urinary tract; sulfadiazine (Fig. 5) is widely used in the treatment of burns, while the combination sulfadoxine with pyrimethamine (Fansidar, Fig. 5) is used in antimalarial therapy. In addition, the azo linkage, the reduction of which released sulphanilamide from Prontosil, is also employed to join smaller therapeutic molecules for subsequent release (i.e. again pro-drug action) after metabolism, typically in the human intestine, e.g. Sulfasalazine (Fig. 5).

6. Increasing dye efficacy – photosensitisers

The majority of dyes are simple from an electronics viewpoint. Promotion of an electron to a higher level via the absorption of one wavelength of visible light, with the remainder of the light transmitted, gives the sensation of colour. The electronic transition is transient, the promoted electron returning to the ground state rapidly (Fig. 6). However, with some dye chromophores, the promoted electron remains in the excited state for a relatively long time (microseconds). This allows other events, such as electron or energy transfer to occur to the molecule's environment – in other words, light promotes activity, and this is the basis of *photo*dynamic therapy. Reactions here usually involve oxygen species such as superoxide, the hydroxyl radical, and singlet oxygen. The latter of these is known, due to its high reactivity, to be destructive in biological media, causing cell damage/death. Photosensitisers which can be targeted at non-economic cells (pathogenic microbes, tumours, etc.) can thus be activated *in situ* with consequent cell inactivation. The anti-tumour application, usually known as photodynamic therapy (PDT), has been in clinical use for over 20 years whereas the antimicrobial application, photoantimicrobial chemotherapy (PACT [18]), is not yet in widespread use.

While it is true that PDT is based on porphyrin pigments, the lead compound used in the development of PACT is the phenothiazinium dye methylene blue, Ehrlich's antimalarial from over a century ago. In terms of research, there is a considerable body of work here devoted to drug design and discovery, more akin to that seen in mainstream antimicrobial drug research. As with the early pioneers, dye molecules are being designed for the purpose of killing microbes [19].

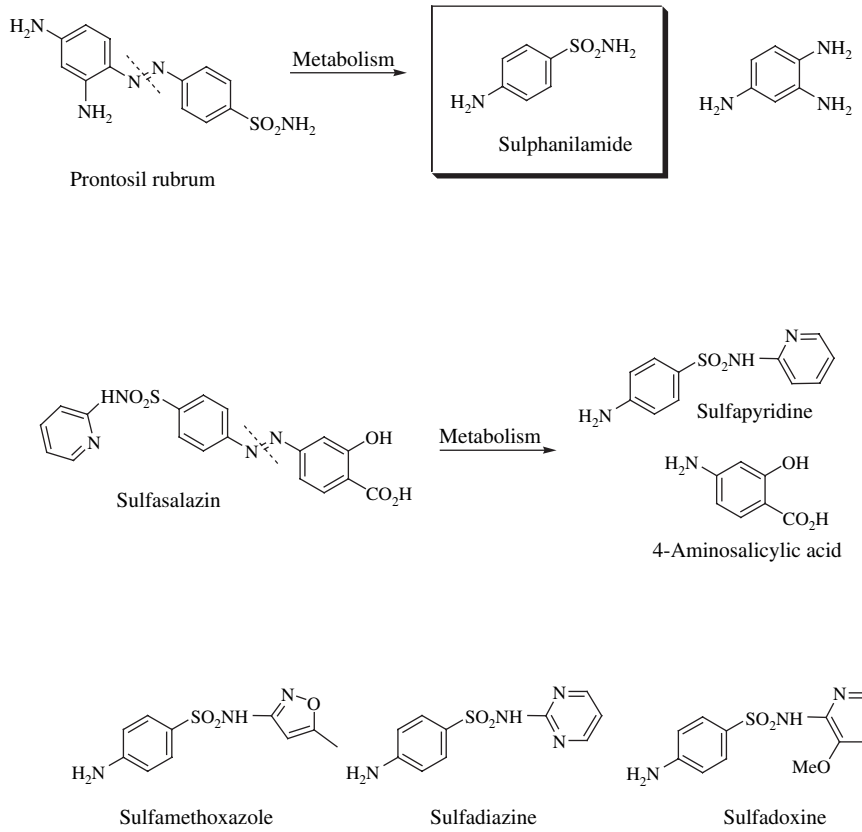


Fig. 5. Azo dyes, Prontosil and the sulfonamides.

The drawback with photosensitisers, by definition, is that they require light for activation and this limits their useful application. In the photodynamic therapy of cancer tumours of the skin, ENT, lungs, brain and GI tract are accessible to light sources of various types. In terms of the photoantimicrobial application, this is at present likely to be limited to skin and soft tissue infections. However, the efficacy of the approach to conventional drug-sensitive and drug-resistant microbes (such as methicillin-resistant *S. aureus*, MRSA) [20–22] offers significant utility in the present climate of increasing healthcare and community infection.

Other areas of useful disinfection include the application to blood products. This area of medicine includes the provision of blood fractions (red cells, platelets and plasma) and clotting factors derived from blood donation to healthcare concerns, for example, for replacement during surgery, or for the treatment of clotting disorders. Plainly, the blood product must be sterile to avoid potentially harming any recipient. This became most evident in the number of patients suffering from HIV infection after a blood transfusion in the 1980s, but there are various pathogenic microbes which might be transmitted, from low grade bacterial infections to AIDS and malaria. The use of photosensitising dyes which can target DNA-containing pathogens in the donated blood sample is a clean method of eradication. Perhaps unsurprisingly, the major product on the market in this area uses methylene blue for the photodisinfection of blood plasma [23]. Considerable research is underway aimed particularly at developing photosensitisers for use in red cell concentrates [24].

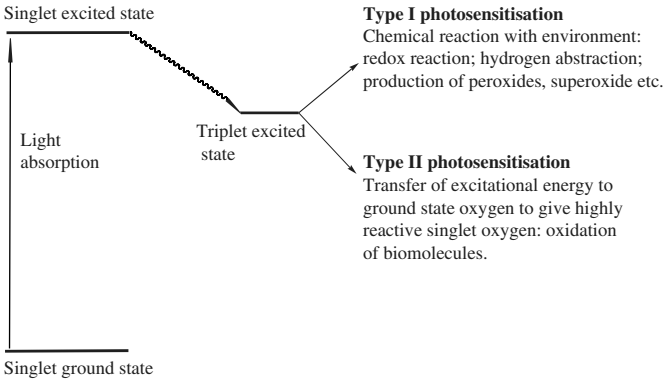


Fig. 6. Photodynamic action.

7. Old dyes and new tricks?

Until quite recently, in the post-penicillin period, the belief existed that microbial disease was either conquered or soon to be so. Unfortunately, the belief was so widespread that the continued development of new antimicrobials has not kept pace with microbial evolution, and, even worse, our use of valuable antimicrobials has been squandering rather than circumspect and has even increased the pace of microbial mutation and change. Consequently, there is a global problem of microbial drug resistance – both in healthcare and in the community, leading to increased morbidity and mortality.

To combat this, researchers have looked outside traditional drug types for an answer, for example to the now ubiquitous MRSA, and this has included the use of dyes. The original Gram stain dye, crystal violet, has been employed to combat MRSA in hospitals in Japan [25]. A phenazine dye, clofazimine, useful in the treatment of Hansen's disease (leprosy) has also been proposed for the treatment of common drug-resistant hospital infection [26].

As noted above, the original synthetic antimalarial, methylene blue, is again in clinical trials in Africa [7]. In addition, there has been considerable research carried out using methylene blue and its congeners as lead compounds in the development of new antimalarials [27,28]. The rise in resistant malaria has also seen the introduction of an acridine derivative, pyronaridine (Fig. 3). This drug was developed in China from earlier acridine work and is currently used in areas of chloroquine resistance [29].

Similarly, recent concerns about the prion disease new-variant Creutzfeld-Jacob Disease (nvCJD) led to the use of dyes as lead compounds. Similarities between the brain lesions found in the disease and amyloid plaques suggested the use of amyloid stains such as Congo red in this respect. Unsurprisingly, drugs derived from this type of azo dye, e.g. Suramin (Fig. 1) have been proposed for anti-CJD therapy [30]. Other dyes and dye-based compounds, such as mepacrine and suramin have also been shown to have favourable anti-prion characteristics.

8. Conclusion: why are not dyes used more?

The obvious property of dyes – their *raison d'être*, of course – is the propensity to impart colour. The use of dyes as drugs in human subjects obviously offers the potential for skin coloration – indeed this is the case with some anticancer drugs such as the anthraquinone derivative doxorubicin. However, the degree of coloration depends on both the route of administration and the pharmacokinetics of the dye/drug involved. The (yellow) acridine antimalarial drug mepacrine does cause yellowing of the skin after oral administration for several days, but the equally yellow acriflavine does not, as it is excreted rapidly. The physicochemical factors governing this behaviour are well understood, so it is possible to ''design out'' unfavourable properties such as skin staining. The local administration of dyes, for example in the photodynamic disinfection of a wound, might stain the patient at the wound site, but not elsewhere.

The other major worry for the pharmaceutical industry is that some dyes have been shown to cause mutation *in vitro*. Dyes such as acriflavine cause frameshifts in the nucleic acid of yeast cells and give a positive Ames test (nucleic acid distortion in the bacterium *Salmonella typhimurium*). However, there is little evidence for mutagenicity or carcinogenicity in human subjects – surprising, given the amount of acridine-based drugs used in the population during the 1930s and 1940s. Other dyes, such as benzidine-containing azo dyes are known to be cancer-causing in humans, and obviously such compounds would be avoided. However, proper design of dye analogues can remove these traits without loss of proper activity. In addition, effective ''dye therapy'' of an otherwise fatally diseased patient could be justified in terms of sufficiently increased life expectancy, even with a dye which does not pass all of the regulatory tests. Given the huge problem of bacterial drug resistance in particular, there is much to recommend deeper investigation of dye-based drugs.

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