Anti-infective Dyes in the Time of COVID

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Abstract

The phenomenal global upheaval caused by SARS-CoV-2 has produced amazing responses from science and healthcare, particularly in the rapid realisation and production of vaccines. However, until early 2020 global infection control research was highly focused on rapidly increasing rates of conventional antimicrobial resistance (AMR) and the supply of drugs to counter this. Antimicrobial dyes have been suggested by various authors for inclusion in this effort, usually with little return from responsible authorities, and normally on the basis of post-treatment staining or potential toxicity, but this does not deny the fact that such dyes, particularly with photoactivation, are the only class of agents with pan-microbial activity – i.e. against each of bacteria, viruses, fungi and protozoa – regardless of the organism's drug resistance status. Conventional antibacterials, antivirals etc. usually demonstrate activity against one particular section of pathogens only, and disinfectants such as chlorhexidine or benzalkonium salts are too toxic for internal use. This perspective reflects both the background utility of antimicrobial dyes and ways forward for their inclusion in 21st Century infection control protocols.

1. Introduction

In the 21st Century, infection control has become considerably more important in the public conscience. This is, in the main, due to the phenomenal, all-encompassing effects of the coronavirus pandemic caused by SARS-CoV-2, aka COVID-19 [1]. Unfortunately, the fact that the virus in question was unknown before late 2019 meant that much of the world's population has been susceptible to infection. Furthermore, the speed of infectivity and mutation of the virus has allowed no time for the development of conventional antiviral therapeutics, and healthcare authorities have relied on the rapid development of biologicals (i.e. vaccines) to combat further waves of the pandemic [2].

It is unfortunate that the admirable collaborative efforts made in science and medicine since early 2020 mean that the ongoing requirements for advancing infection control outwith COVID-19 have scarcely been met. Global mortality forecasts of 10 million per annum by 2050 caused by antimicrobial resistance (AMR) [3] originally spurred efforts in the search for new antimicrobial agents to combat this threat, but much of the research effort was, perforce, turned to the battle against the coronavirus. Obviously, the original threat to infection control from AMR remains and can, indeed, only be increased by the diversion of resources caused by COVID-19, in addition to the decrease in useful funding which is always associated with the consequent global financial downturn.

In addition to the search for new antimicrobial agents, it has been admitted that *new approaches* to the problem of AMR are also required. When this admission first began to appear in articles and press releases in 2017, groups involved in "non-antibiotic" drug research were initially enthused. However, this was a fleeting sensation, since it was rapidly apparent that the new approaches envisaged merely indicated biological, rather than chemical, drug-centred approaches, *viz.* vaccines and phage therapy [4], as ever reflecting the highly conservative nature of modern medicine.

Among the groups working at the periphery of infection control are those concerned with light-activated chemistries, both exo- and endogenous, i.e. relying on the photoexcitation of administered or *in situ* (bio)molecules, respectively, to produce a local and highly-controllable killing effect (Figure 1) [5,6].

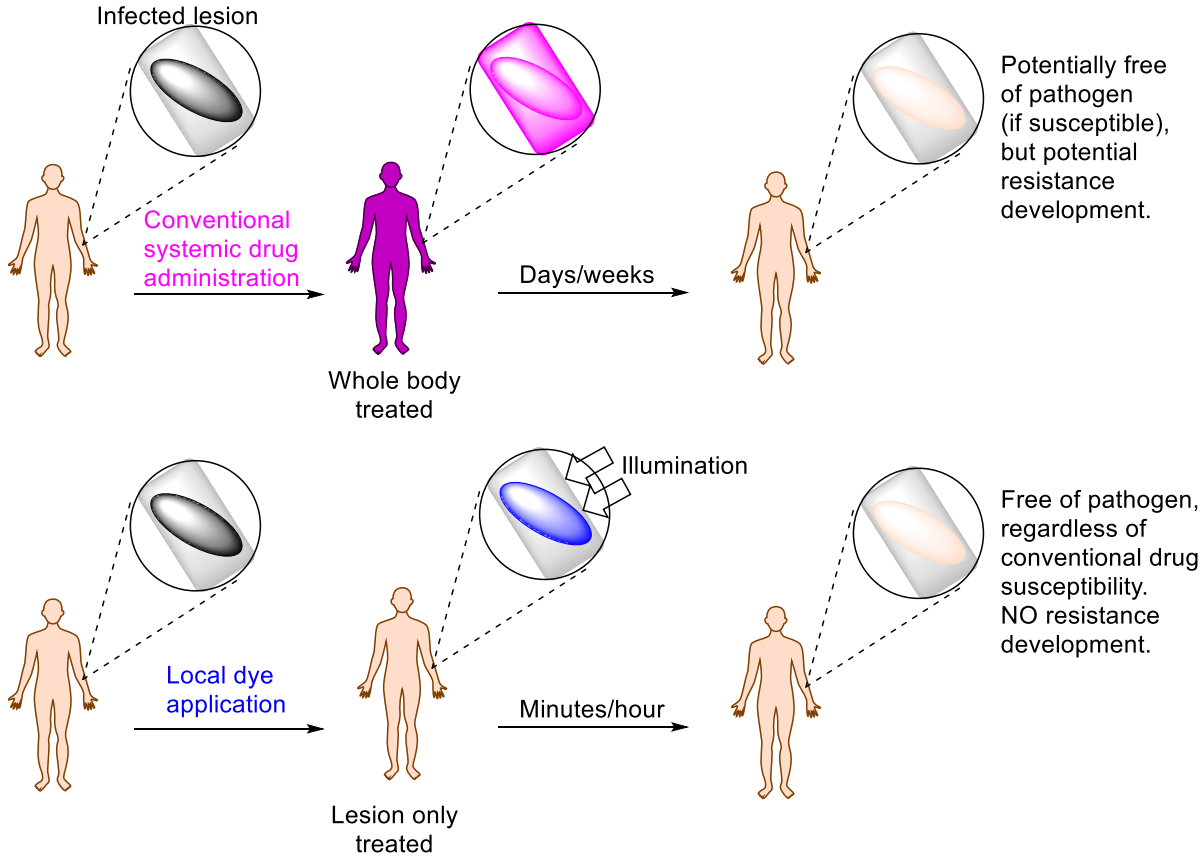


Figure 1. Conventional and light-activated approaches to infection.

The body of research produced in this area has its origins mainly in biological staining and the work of Ehrlich, Gram, Koch and other early scientific pioneers [7]. For this reason, light-activated technology aimed at infection control is often discounted on the basis of antiquity and/or "dye toxicity" (whether evidential or not). However, objective assessment demonstrates the potential of the approach across a huge range of pathogenic (disease-causing) targets, importantly regardless of conventional resistance status, and for these aspects alone, far greater investigation and testing should currently be underway. This is not the case.

The following perspective covers the utility of small colourant molecules in killing or otherwise inactivating the microbial species responsible for infectious disease in both *Homo sapiens* and his animal - food or domestic - cohabitants. The focus is therefore on synthetic dyes, rather than on endogenous biomolecules. Some examples covered in the text are shown in Figure 2.

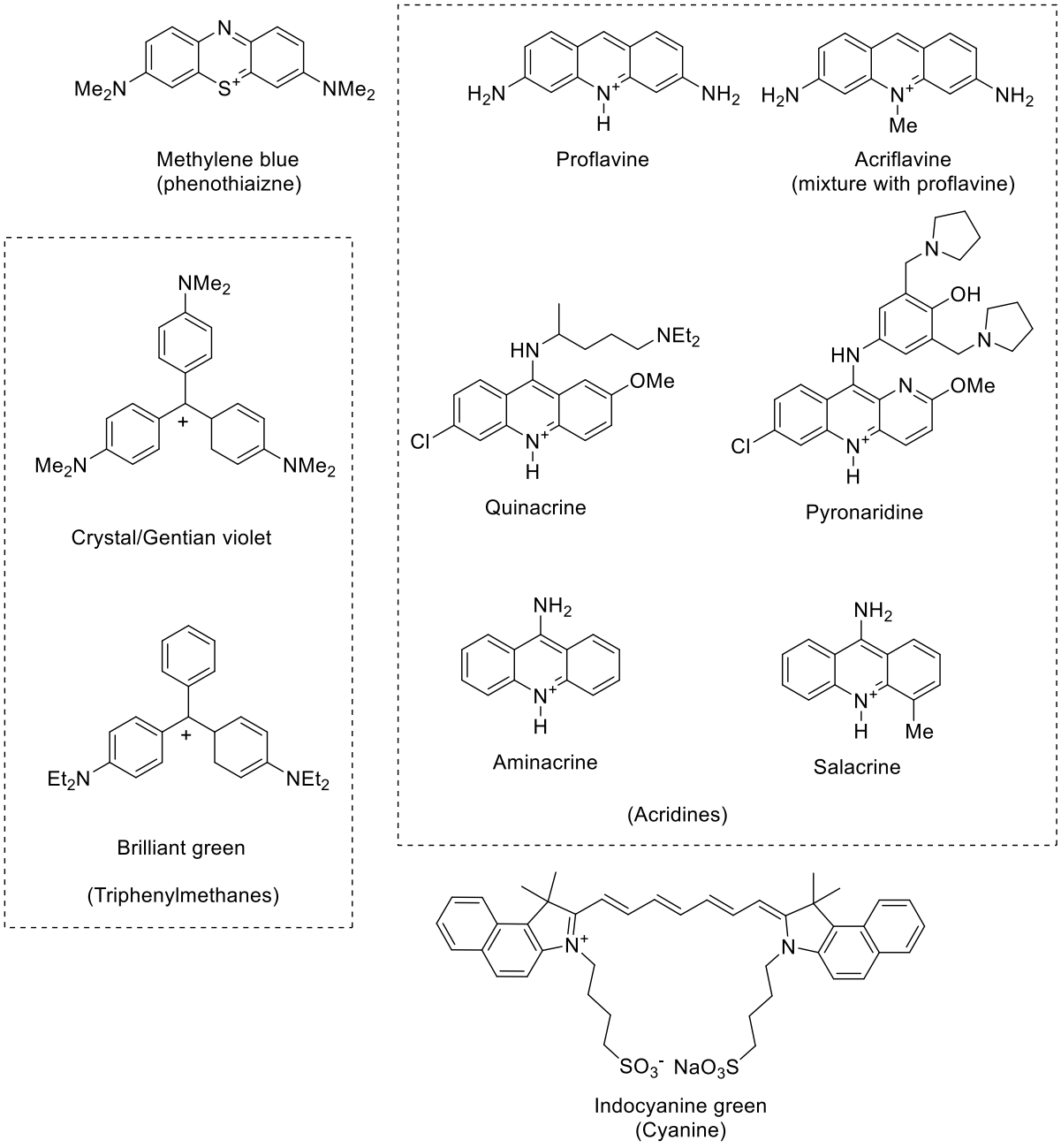


Figure 2. Dyes and dye derivative used in medicine.

2. Dark and light – the duality of death

The research carried out by the above-mentioned Ehrlich, Gram and Koch, among others, in the late nineteenth Century utilised newly-accessible synthetic dyes either to stain cells for microscopy or, by extension, to inactivate cells. This opened up both disease investigation and, ultimately, therapy. In addition, gross structure-activity relationships were developed and later refined, and from this modern chemotherapy emerged. Gram's definitive bacterial classification method, Koch's identification of the cause of tuberculosis and Ehrlich's clinical cure of plasmodial malaria all date from the end of the 19th Century [8].

The first report of *photodynamic* action (i.e. caused by light activation – see above, and Figure 3) associated with synthetic dyes in a biological system was by Raab in 1900, relating to his experimentation on *Plasmodium caudatum* [9]. Conversely, the first report on specifically photobactericidal activity (i.e., resulting in bacterial cell death) concerned the phenothiazinium dye methylene blue (MB) in 1908 [10].

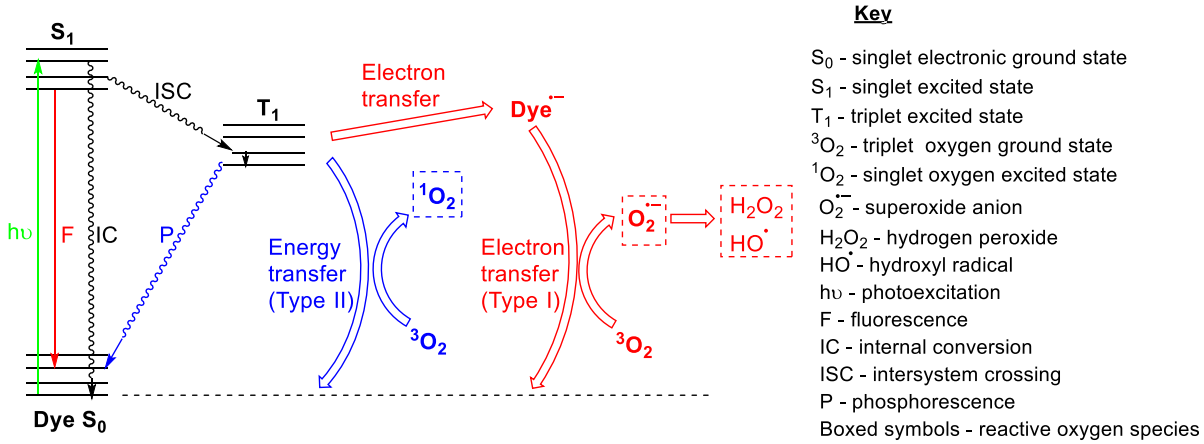


Figure 3. Light-activated dye production of reactive oxygen species (ROS)

Given that the acridines, later used by Ehrlich, as well as the triphenylmethane dyes utilised by Stilling (1890/1)[11,12], Churchman (1912/3)[13,14], and later by Browning (1913 onwards)[15], are all also photosensitisers - although this was not understood at the time - it is interesting to speculate on how much of the conventional activities of these dyes might actually be attributed to photodynamic action due to incidental illumination. This is likely to be a significant positive in terms of clinical acceptance, given that there is no apparent literature evidence that environmental light exposure experienced by patients (liberally) treated with acriflavine or crystal violet for skin wounds or ringworm (tinea capitis, a fungal skin infection), respectively, led to any deleterious effects. Both of these compounds - and other small molecule colourants – were in wide topical use until past the middle of the 20th Century in Europe and North America. From a more conventional antimicrobial viewpoint, the acridine drug quinacrine (aka mepacrine, atebrin) was a widely used antimalarial, both in the treatment and prophylaxis of disease [16]. This was well-known to localise in the skin, often giving the recipient a yellowish colouration with respect to the skin and eyes [17]. Again, there is nothing apparent in the medical literature regarding reported tissue photosensitisation or clinical sequelae, and this is also the case for the currently used benzo[*b*]naphthyridine analogue pyronaridine [18].

Given the established beneficial effects of antimicrobial dyes, it is frustrating to those working in the field that the merest mention of a dye in connection with its proposed clinical use normally elicits many of the graver symptoms listed in modern pharmaceutical packaging among medics or healthcare managers, very few of whom have any direct experience of the biological – *or clinical* - effects of the dye in question. The shadow of *in vitro* DNA experimentation looms very large indeed!

3. Nucleic acids – the duality of targeting

There is very little point in arguing that there is no significant interaction between planar cationic molecules and nucleic acids *in vitro*. Lerman's and other publications [19,20] in the 1960s concerning proflavine fluorescence were both immensely useful from a microscopic sensing viewpoint and subsequently (and consequently!) highly detrimental to most researchers seeking to cause *useful* cell damage or cell death using similar molecules. There is, of course, a sound scientific logic to this detriment, at least for those wishing to build the basis of an anti-drug use argument. However, the danger lies in the reliance on extrapolation (normally from *in vitro* data to *in vivo* prediction) to provide and nourish the argument, particularly where there is a lack of evidence.

The major missing piece of evidence here is that of host tumours caused by anti-infective dye usage since this would be expected as a consequence of DNA mutation caused *in vivo* by the dye. As mentioned above, the incidence of the medical use of the acridine class alone is considerable, from battle injuries in World War 1, through civilian use between the wars for a variety of infections and back again to battle injuries in World War 2, with the addition of enormous contemporaneous use of the acridine antimalarial drug mepacrine in the Pacific Theatre. However, there is no report of groups of ex-servicemen subsequently presenting with a certain type of tumour, leukaemia or lymphoma as a result [21]. Given the amount of study and reportage regarding the fate of military veterans involved with post-war nuclear bomb tests, the absence of literature concerning anti-infective dye-related side effects in much larger cohorts suggests an absence of such sequelae.

This is, of course, in direct contradiction to the results of many *in vitro* tests. The intercalation of simple planar cationic molecules of sufficient size between consecutive base pairs in DNA helices is easily demonstrated, and not limited to dyes. Albert's epic study of acridines resulted in several excellent clinical anti-infectives, but his understanding of the structure-activity relationships involved enabled him to show that non-heteroaromatic analogues (e.g. based on anthracene) could function via the same structural paradigm [22]. It was poor timing, indeed, that two of Albert's non-staining derivatives, 9-aminoacridine and 9-amino-4-methylacridine (Aminacrine and Salacrine, respectively), would presumably have made more impact had they not been introduced in 1945/6, in the same period as penicillins G and V [23].

Later studies have demonstrated that sterically hindered aminoacridines – e.g., bulky alkylated proflavine analogues, do not intercalate with DNA [24]. This suggests an interesting direction for future work.

4. Industrial production

The dye industry has generally had a poor reputation in terms of cleanliness, environmental and ecological considerations. Dye synthesis or formulation laboratories are usually heavily stained as a consequence of their basic purpose, so it is of no surprise that manufacturing facilities often appear

to be far worse, if only due to the scaling-up process. In addition, commercial dye samples are not usually pure, although this is often by design in textile dyes, due to improved solubility and dyeing properties.

Such a sullied reputation clearly does not help when human exposure to dyes is suggested. Neither is this improved by the toxicity of several chemicals used in the production of dyes. For example, exposure to the azoic dye precursor benzidine is associated with an increased risk of human bladder cancer [25].

The Ames test is a biological assay often used to indicate the potential for deleterious effects on DNA, changes being caused by the test subject to certain strains of the bacterium *Salmonella typhimurium*. Interaction of the Ames test subject with DNA or its attendant enzymes can lead to alteration of expressed "daughter" DNA, i.e. mutation. This is not a direct guarantee of cancer causation in mammals, but, obviously, planar cationic dyes will interact with DNA and so most provide a positive Ames test. However, it should be pointed out that members of the widely used fluoroquinolone antibacterial class, e.g. ciprofloxacin, also interact with DNA and provide positive Ames tests [26].

Producing a dye that is intended for human or veterinary use in the 21st Century is thus a difficult prospect, particularly from the point of view of progressing through the various regulatory authorities entailed. However, any dye intended for such use can only differ from a conventional antibacterial drug, for example, in that it is a coloured compound. This means that it must satisfy the usual criteria pertaining to materials supply chain, compound purity and stability, in addition to toxicology/mutagenicity and drug efficacy. Such criteria could not be satisfied if the dye were produced under the same conditions as conventional colourants, e.g. for textiles, which therefore also precludes the use of dyes which have been produced by conventional industry and then purified. Consequently, medical dyes such as those under discussion here would require *de novo* production facilities with the same standards as those set for conventional pharmaceuticals.

5. Changing the *status quo*

There is, of course, a considerable period of time between the amazing breakthroughs made in the various scientific disciplines underpinning the realisation of even an elementary chemotherapy and modern, 21st Century infection control. The use of what were once considered to be *antiseptic* dyes – i.e. dyes which could be applied to cure local infection and prevent its development into systemic disease – is nowadays generally avoided in affluent countries. The scientific justification for this is normally either weak or absent and - among scientists at least - this should be unacceptable. However, healthcare is rarely run by scientists and it is even rarer to see them in government.

As an example, consider the use of the acridine compound proflavine and the triphenylmethane derivative brilliant green in wound therapy. This began in earnest with their application by Browning in gunshot or shrapnel wounds during the second half of World War 1 [27]. Since most fatalities during the conflict were caused by post-injury bacterial infection, Browning's approach was logical and, as it turned out, successful, leading to the use of these antiseptics also in civilian medicine during the 1920s and 30s [28-32]. As an aside, it is interesting to note that in proving the efficacy of proflavine in antisepsis in 1918/19, Browning and his main co-worker Gulbransen employed a streptococcal peritonitis model in the mouse [33], as Domagk later did in his breakthrough work on Prontosil. The latter research, of course, eventually realised the sulphonamide antibacterial class [34].

Proflavine, normally applied as the hydrochloride salt, is bright yellow in solution. Both proflavine and brilliant green produced staining of local host tissue during use. When proflavine, or the related mixed compound acriflavine, was given orally, for example in the treatment of gonorrhoea, the urine also assumed a deep yellow coloration [35].

This contrasts starkly with the use of the initial sulphonamide, sulphanilamide, or with the principally-used introductory β-lactam agent benzyl penicillin (penicillin G). Sulphanilamide was applied locally and orally, while benzyl penicillin was usually administered intravenously. In neither case was there any tissue coloration, and in both cases, regardless of the administration route, the antibacterial effects were normally greater than those arising from proflavine use.

Given such results, why should antiseptic dyes such as proflavine still have been considered for infection control applications? Contemporarily, proflavine was, in fact, employed throughout World War 2 as a local antiseptic [36], often as an adjuvant to sulphathiazole as they form a neutral salt [37], but this may be seen in terms of exigency during wartime. Post-war, its usage decreased. While it must be admitted that antibacterial drugs such as benzyl penicillin and its congeners are more effective against what might be termed internal infection, proflavine remains available and still appears in national formularies for infected wounds and burns [38].

Really, this is not a case of comparing like with like. The conventional approach for the administration of antibacterial drugs is usually by mouth or by injection. Low toxicity to the host allows the distribution of the drug throughout the system and sufficient concentration by the target organisms to achieve the requisite level of killing or inhibition, allowing the host's immune response to take over subsequently. Administering dyes such as proflavine/acriflavine via either route cannot achieve such results in many cases due to its highly hydrophilic nature – i.e., blood serum concentrations are rapidly diminished by the standard excretion process via the urine. Indeed, this explains the sparse (though not absent) reporting of acriflavine successes in gonorrhoea therapy via systemic administration [39]. Direct instillation (or application) represents a far more logical approach, but this may only be used where there is an established locus of infection.

Furthermore, where there is a definite locus, local application *should* be encouraged. One of the tragedies of the administration of our conventional antimicrobials is that this usually takes little account of drug resistance development caused by collateral effects on endogenous flora (the microbiome) during systemic administration. With a dispersed microbial target, as in septicaemia, such collateral damage is completely excusable because of the seriousness of the infection, but where the target is concentrated to a local volume, it is surely not.

Local antiseptics have not generally been used internally since the advent of systemically-administered antibacterial agents. However, in view of the contemporary - and growing - degree of bacterial resistance in particular, this situation should be altered where the pathogen is locally susceptible.

Microbial drug resistance (i.e. not limited to bacteria) has been reported for local antiseptics for decades, although it is not complete. Like Browning's flavine therapy, local treatment with quaternary ammonium compounds (QACs), such as benzalkonium chloride and bisguanidines (BGs) such as chlorhexidine salts employs cationic agents. It is established that these cations are often combated via the overexpression of efflux pumps in bacterial cells – the gene responsible in *Staphylococcus aureus*, for example, *QacA*, is known as the antiseptic resistance gene [40]. Furthermore, such overexpression of efflux pumps can confer cross-resistance to conventional antibacterial agents such as the fluoroquinolones [41].

Consequently, the suggested re-introduction of cationic antiseptics in order to conserve conventional antimicrobial drugs for use against more serious systemic disease may seem something of a false move. While this may be the case with QACs and BGs, light activation of antiseptics such as proflavine, crystal violet or brilliant green, leading to photodynamic action would negate the effects of drug efflux. The non-specific reactive oxygen species (ROS) produced degrade efflux proteins or, if the dye is effluxed, ROS produced at the cell exterior cause major damage to the bacterial outer membrane and/or cell wall. Furthermore, the dye concentration required for effective photodynamic action is much lower than that for conventional antisepsis using QACs and BGs.

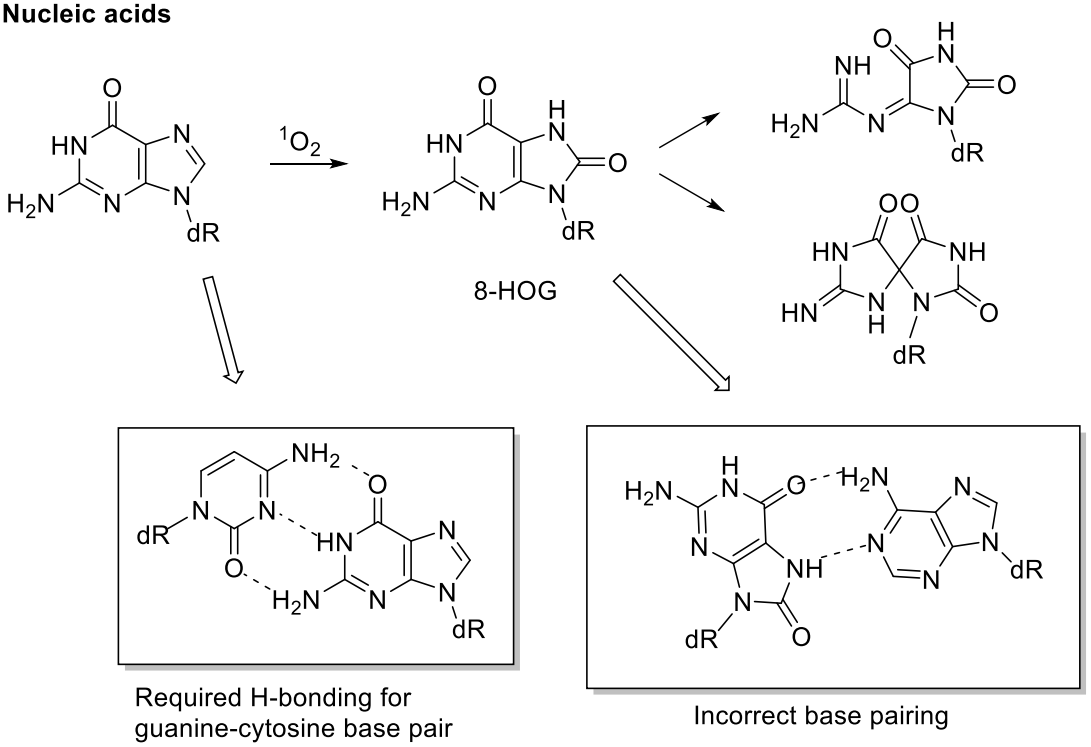
5.1. Photodynamic action

How can cationic photosensitising dyes be so much more efficient than standard antibacterials and antiseptics? The key to this lies in the production of the above-mentioned ROS immediately following illumination.

Firstly, it should be remembered that the target microbial cells are very much smaller, simpler and less well protected than host cells, whether these are human or animal in nature. This size and simplicity mean that damage may be caused easily, rapidly and with little or no chance of remediation by the organism.

The reactive oxygen species produced as a result of photosensitisation include the superoxide anion (O_2^-), hydroxyl radical ($HO^\cdot$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$, Figure 3). Generation of such species leads to oxidative reactions with a variety of biomolecules, as exemplified in Figure 4 for singlet oxygen with guanine and aromatic peptide residues – i.e., DNA and protein targets.

(i) Nucleic acids



(i) Peptides/proteins

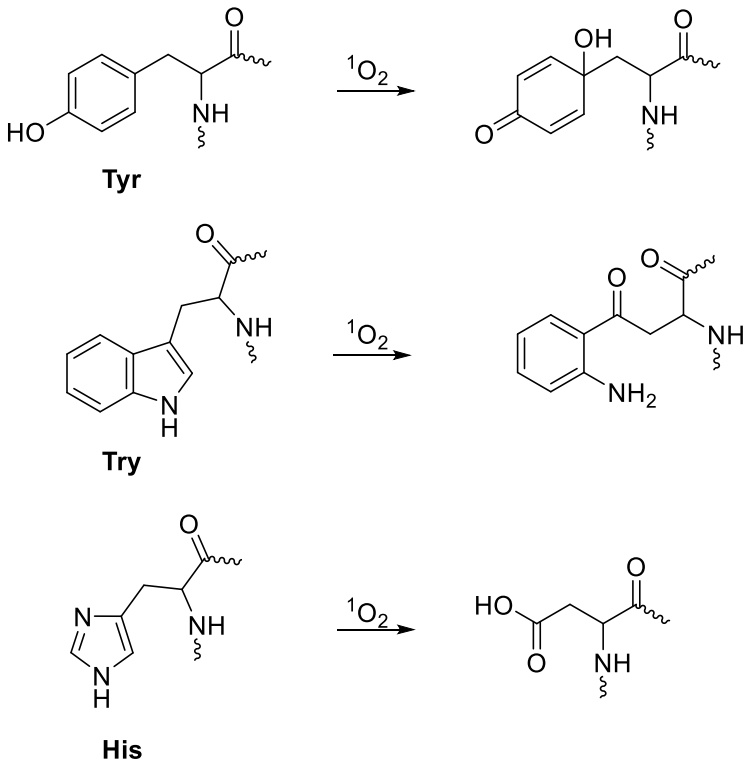


Figure 4. Biomolecular damage caused by singlet oxygen for guanine and peptide residues. Key: dR – deoxyribosyl unit; 8-HOG - 8-hydroxyguanosine; Tyr – tyrosine; Try – tryptophan; His – histidine.

As with other ROS the molecular alterations resulting from reaction with singlet oxygen cause loss of function at the altered moiety, this loss therefore being potentially structural, enzymic or genetic/translational, all of which can be catastrophic in simple, microbial cells. In addition, given that all microbial targets are constructed from the same biomaterials, the impression of efficacy across microbial types, rather than *solely* bacteria *or* fungi *or* viruses etc., is more easily appreciated. Figure 5 shows the difference in outcome between methylene blue interactions with DNA under dark and light conditions, underlining the catastrophic effects on microbial survival following the latter approach.

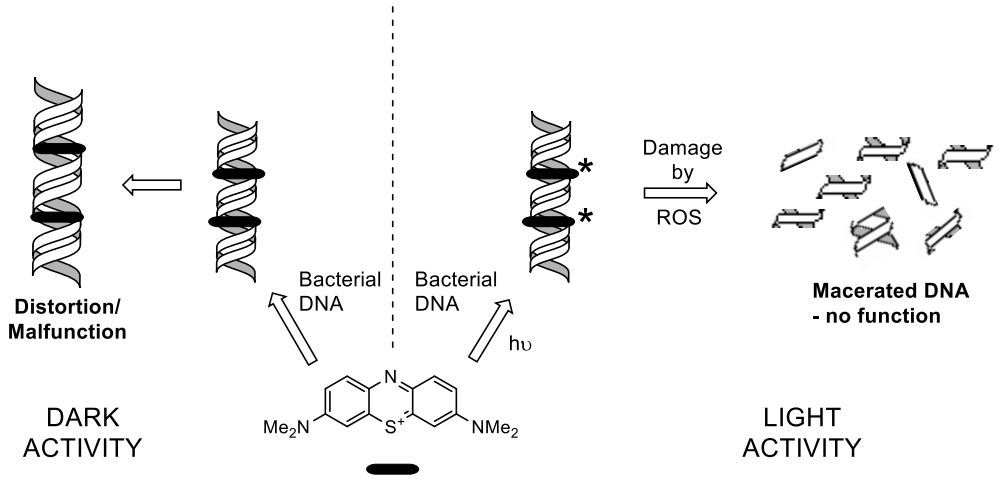


Figure 5. Conventional and photodynamic action underpinning one approach to antibacterial activity at DNA for methylene blue. * indicates photoexcited molecule.

Further to this, the purely basic chemical reaction paradigm entailed by the photodynamic approach, as opposed to the exquisite targeting demonstrated by specific enzyme inhibitors, allows photodynamic attack at any phase of microbial life – lag-, log-, stationary- or death phase in bacteria, for example - enzyme activity is not a requirement. As noted above, the combination of multiple modes and various sites of action means that microbial colonies cannot produce and express sufficient useful and synchronous mutations to confer resistance to this approach [42]. This would include a pan-conventional resistance mechanism, such as cell wall thickening in *Staphylococcus aureus* or decreased membrane permeability due to porin absence in *Pseudomonas aeruginosa*, since oxidative attack is possible from both inside and outside the target cell, which guarantees efficacy.

The demonstration of the broad-spectrum efficacy of the photodynamic approach to bacterial resistance was recently reported by Sabino *et al*. In this work, the authors utilised a standard treatment protocol with methylene blue and red light against conventional drug-susceptible and -resistant strains of the ESKAPE bacteria (the group of bacteria responsible for most global healthcare-associated infections, such as *Enterococcus faecium*, *Staphylococcus aureus* and *Klebsiella pneumoniae*). The bacteria used demonstrate all of the main resistance mechanisms (drug target alteration, drug inactivation, overexpression of efflux pumps and decreased cell permeability),

and include the antiseptic resistance gene mentioned above. Using 100 mM of methylene blue and red light, more than a $5\log_{10}$ reduction was observed within less than 75 seconds of illumination [43]. It should be emphasised that this particular study demonstrated rapid success against bacteria which are resistant to over 50 conventional antibacterial drugs, i.e. they are effectively *totally* drug resistant.

The degradation of biofilms also represents another facet of ROS attack at an infected/colonised site which is unavailable to conventional agents [44]. Again, this is facilitated by chemical oxidation, this time at the polysaccharide matrix of the biofilm.

Consequently, the microbial target is rapidly killed or inactivated without increasing the resistance facility in the host's indigenous flora. In addition, the exposure of the host is minimised, both by this rapidity of treatment and also by local illumination only of the infected area or volume. This means that a *photoantimicrobial* effect is only produced by the conjunction of dye, light and oxygen, and this can be simply controlled – any dye escaping from the infection site remains, by definition, non-illuminated by the treatment process. Conversely, conventional antimicrobial therapy exposes the whole individual, with concomitant side-effects both to the host and the microbiome.

5.2. It's in the blood!

There are many routes utilised by microbes to travel between humans or humans and animals. Indeed, animals are still often unconsidered during infection disease outbreak investigations, although this is less likely since the recognition of the links between food animals and humans regarding AMR [45]. In addition, certain animals have long been established as infectious disease reservoirs for what Western Medicine calls *tropical* diseases.

One of the problems with such diseases is that the infective agents are often transmitted by insect bites (e.g., malaria, trypanosomiasis, yellow fever) before becoming bloodborne, which may mean that the recipient does not realise that they are carrying the agent – this will only become apparent when symptoms occur with sufficient severity to require investigation.

However, prior to any potential disease investigations, transfer of the infective agent may occur via the body fluids of the recipient. For example, the transmission of human immunodeficiency virus 1 (HIV-1) via unprotected sex or shared intravenous needles during drug use was unappreciated properly for several years and, in the event, guaranteed the explosive nature of the subsequent pandemic. What was even less appreciated was HIV-1 transmission among haemophiliacs and related users of blood products. Therapeutic materials such as factor VIII, a clotting promoter used by patients with haemophilia and von Willebrand syndrome, is isolated from blood plasma, in turn fractionated from whole (i.e., donated) blood. Collecting blood from donors particularly during the early years of the pandemic, when the nature, and even the identity, of HIV-1 was unknown ensured its presence in the blood supply, especially in countries where payment was made for blood donation. Pooling (combining) of plasma prior to factor VIII isolation exacerbated this problem and resulted in over 4,000 AIDS deaths among haemophiliacs in the USA alone [46].

Clearly other microbial foes can be found in the blood of infected donors – indeed one outcome of the devastation caused by HIV-1 has been that systematic overview of the whole process from donation to transfusion (haemovigilance) is now carried out. Dyes are part of this approach.

As noted above, viral contamination of the plasma supply led to significant mortality among haemophiliacs. For the most part this was due to the fact that the responsible authorities were not

looking for HIV-1 in blood products, the virus being an *emerging pathogen* in the late 1970s and early 1980s. Advances in molecular biology now allow the detection of both known and new/emerging pathogens in blood samples and while this does not solve the problem, it does at least indicate that the problem exists and this is where dyes - usually one dye in particular – play a vital part.

The use of methylene blue (MB) for the decontamination of plasma was first suggested following research carried out at the Walter Reed Institute in 1955 [47], work which was aimed at combating the presence of malarial parasites in pooled plasma used by the US military. Methylene blue was the first synthetic compound to be used as an antimalarial [48] and, because of this, was used as a structural lead in the development of many 20th Century antimalarial drugs [49]. However, the blood decontamination work was based on its photodynamic properties, rather than using it as a conventional antimalarial. Surprisingly, support for the introduction of the MB-photodynamic treatment approach did not reach critical mass until the late 1980s.

The plasma fraction, as noted, is non-cellular – i.e. platelets and red cells are separated off – but can carry smaller particles, such as viruses. Treatment of donated plasma – whether pooled or not – with methylene blue and light allows inactivation or killing of such particles, whether they are bacterial, fungal, viral or protozoal in nature. This is necessary because just as donors do not represent a uniform source, donated blood may contain any of the pathogenic types mentioned above. Indeed, this facility has become more important with the rapid globalisation of infectious diseases, often meaning that sickness due to a pathogen endemic to one part of the world presents in a completely different, distant location. A good example of this is the 1999 outbreak of West Nile Virus (WNV) disease in the north-eastern US [50]. WNV is currently the principal mosquito-transmitted disease in North America, and transfusion-transmission of WNV occurred at the time of the outbreak for the same reason as for HIV-1 almost 20 years earlier. Given its multiple mode of action/site of action, methylene blue phototreatment is effective in inactivating both viruses in donated plasma [51].

The use of methylene blue in plasma decontamination thus represents a generally antimicrobial protocol. Again the multiplicity of attack means that any enveloped virus will be inactivated, regardless of rarity or virulence – e.g. Ebolavirus [52] - and this would, of course, include SARS-CoV-2 [53]. Indeed, such is the activity around methylene blue and COVID, that direct, specific interaction between the dye and the viral infective spike has been determined, leading to the suggestion of MB as an adjuvant (non-photodynamic) therapy to inhibit viral infectivity [54,55].

6. Can we use dyes in infection control in the 21st Century?

As described above, dyes used to treat bacterial infection were replaced first by natural product drugs, such as the early penicillins and tetracyclines, followed by semi-synthetic analogues resulting from structure-activity and drug development programs. However, the *raison d'être* for these early drugs was that they were antibacterial and were well tolerated, for the most part, by patients when provided as systemic therapy. In no case was the mode of action or site of action of these drugs appreciated at the time of clinical introduction. This was established later, when suitable tools (typically enzyme assays) became available, and this approach, along with huge advances in information technology, have provided modern drug discovery with a strong scientific and rational basis.

The fact remains, though, that expensive, modern penicillin equivalents, such as the carbapenems, attack the same site in the developing bacterial cell wall as did benzylpenicillin in the 1940s [56]; glycylcyclines such as omadacycline, clinically introduced in 2018, still acts on the bacterial ribosome, just like the initial tetracycline, aureomycin, in 1945 [57]. Antibacterial drug resistance is encouraged when the same site is attacked over a period of time, resistant forms flourishing as a result of the selective pressure brought to bear by this practice. Very few conventional antibacterial drugs act other than by a single mode/site of action, despite the well-understood mechanisms underpinning drug resistance. Multiple drug-class combinations are effective, since the different drugs target separately, thus killing more efficiently, perhaps even synergistically, and removing the potential for selective pressure. Multiple-drug administration is not common, however.

It would be ridiculous to suggest that cationic dyes used in local antisepsis did not suffer from resistance. As discussed above, they can be removed simply by efflux pumps, so it is pointless to argue for their re-introduction in place of conventional agents, *as* conventional agents. A therapeutic dye such as proflavine is just a molecule, similarly to common antibacterial drugs, such as chlortetracycline or ciprofloxacin. All are xenobiotic substances and microbial cells are generally provided with mechanisms by which they can remove or inactivate such species. Where proflavine and other therapeutic dyes have an advantage is in their ability to absorb visible light energy and use this to promote local reactions with, or energy transfer to, oxygen, leading to ROS formation and non-specific damage to the local cellular environment.

The properly targeted application of this advantage offers localised and controllable antimicrobial activity, without the spectre of AMR development and with activity across the microbial spectrum. The limiting factor is internal light delivery throughout the body of the patient.

Currently, at least, only localised disinfection is possible using photoactivated dyes. However, systemic disease may be treatable in the future via extracorporeal methods – in other words the removal and treatment of a volume of blood, before its return to the body (Figure 6). Septicaemia is a systemic infection where microbes circulate within the bloodstream, eventually causing disease throughout the body and causing organ failure. However, where this is due to bacterial infection, the numbers of bacteria in the blood are relatively low – serious infection presents as hundreds of bacterial cells per millilitre of blood. Such titres are very small compared to those employed by workers involved in testing the photodynamic efficacy of dyes. For example, in virus inactivation data reported during development work on the phototreatment of blood plasma as discussed above, Huang *et al.* reported the inactivation of 2×10^6 plaque-forming units/mL of dengue virus after a five-minute red-light illumination with 1 μM methylene blue [58]. Where the infecting bacteria are conventional drug-resistant, treatment options for septicaemia/meningitis/sepsis are very limited, so the photodynamic approach is worth further investigation.

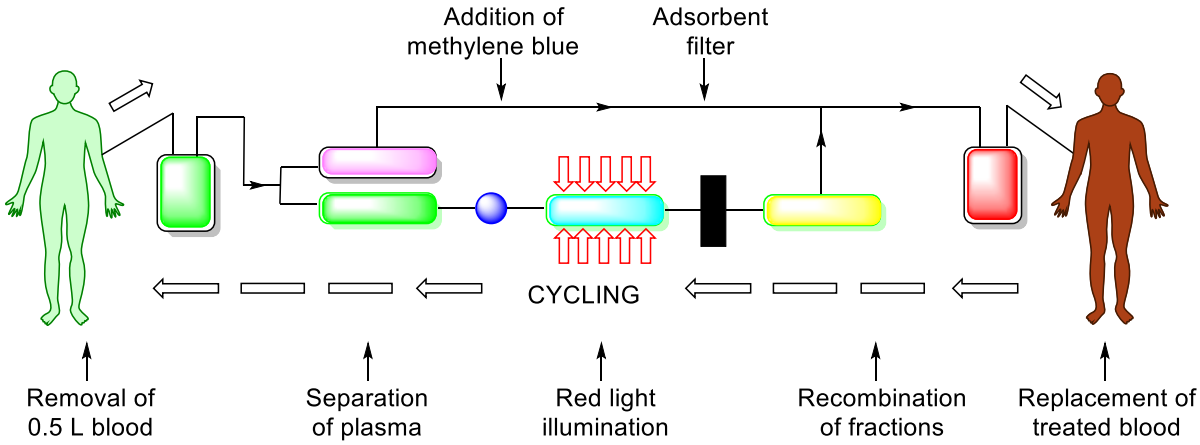


Figure 6. Dye-based extracorporeal photodisinfection.

Equally, in terms of host anatomy, it should also be remembered that localised disinfection does not mean merely external surface treatments, although the dermatological field is an obvious candidate, just as the treatment of skin tumours was, and remains, a mainstay of anticancer photodynamic therapy [59]. *Localised* refers to the physical situation of the infecting/colonising microbial population, and this can be treated anywhere in - or on - the body, either directly, or employing endoscopy and fibre optics where required.

Currently, the main clinical use for dyes lies in oral disinfection and this is light-activated. Typically, methylene blue and red light are employed in dentistry against local infections including periodontitis, endodontitis and peri-implantitis [60-62]. This provides rapid disinfection and offers an alternative to (slower) conventional antibacterial therapy. However, while this approach is both useful and impressive, it remains the province of dentists. In terms of light activation, there has been little organised movement from the mouth into the rest of the body.

Tonsillitis might also be considered, if peripheral, as an infection of the oral cavity. Like those mentioned above, it constitutes a localised infection, in this case mainly in the exterior portion of the tonsils. While most cases of tonsillitis are associated with viral aetiology, the remainder are mainly bacterial and their situation is usually within easy reach for both dye application and light activation. Again, this would allow rapid bacterial killing without harm to the microbiome, and it should be recalled that conventional therapy requires 5-7 days of, e.g., orally-administered amoxicillin which *does* damage the microbiome [63]. In addition, many of the viruses implicated in tonsillitis are enveloped and thus also susceptible to the photodynamic approach, as discussed above with respect to plasma disinfection.

The direct, local treatment of tonsillitis using methylene blue and red light could thus be achieved in a straightforward manner, delivered via general practice nursing staff. This would be inexpensive with regard to the dye and light and, more importantly would neither add to the problem of drug resistance nor use up valuable conventional systemic antibacterial resources which could therefore be conserved for more serious disease.

The direct approach may also be used for less straightforward presentations in the same vicinity, for example where there is chronic infection or conventional drug resistance. Thus, infection of the sinuses, middle ear, adenoids, pharynx and larynx is, at least, targetable (Figure 7a). Similarly, this

approach can be extended to respiratory infection, as shown in Figure 7b. The utility of endoscopy is key in such cases, being able to deliver both dye and light with high accuracy. There is also the option to employ trans-thoracic light delivery in lung disease, although this would only be realistic with near-infrared absorbing dyes. Given the significant current morbidity and mortality due to drug resistant pneumonia, as well as the increased occurrence of drug-resistant tuberculosis, such an approach is worth pursuing.

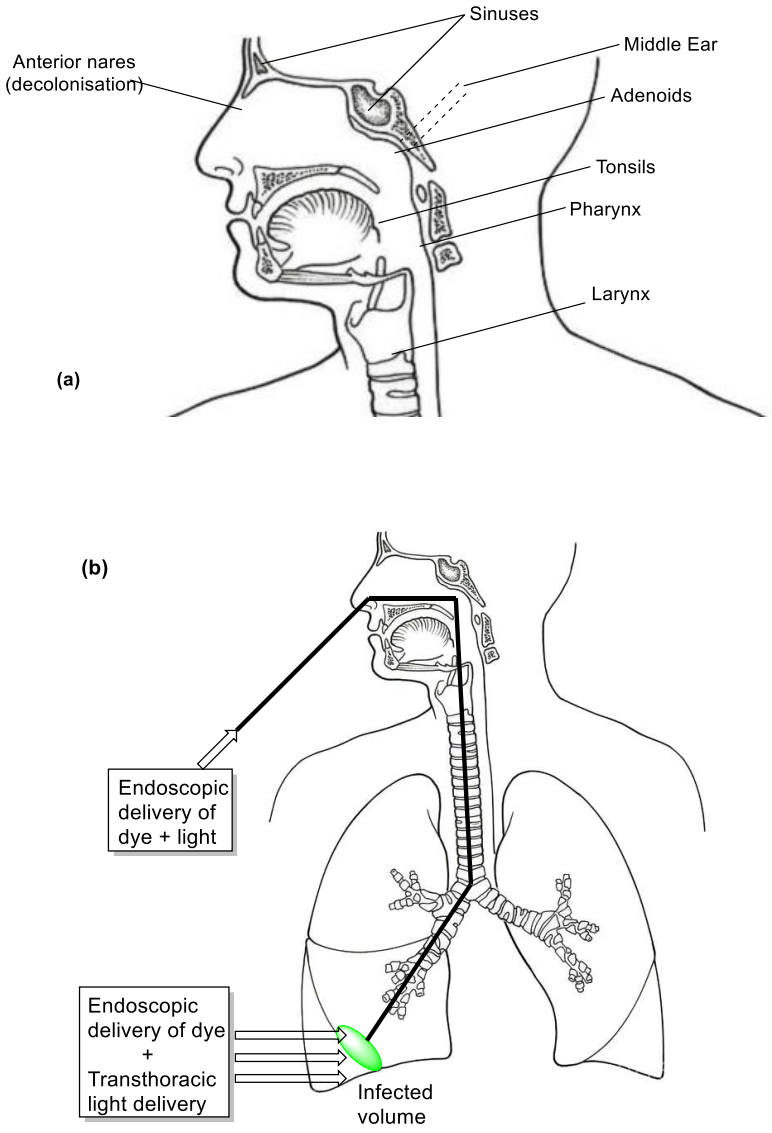


Figure 7. Locally-targeted approaches to the photodynamic treatment of (a) ear, nose, throat and (b) pulmonary infection.

Bacterial tonsillitis, if not resolved, can lead to infections of the respiratory tract, such as pneumonia and further to septicaemia and meningitis. This can be the case if either the primary infection is not treated, or is treated ineffectively – e.g., halting treatment too early. Conventional drug resistance is, of course, also a cause of treatment failure, and in such cases, secondary and further infections

become more difficult to treat, often requiring hospitalisation. Early and effective therapy, as described above, is therefore essential.

Furthermore, there is the problem of microbial – and thus disease – transmission. As an example, bacterial and viral meningitis is often spread between individuals in close proximity due to the presence of the responsible microbes in water droplets in the upper respiratory tract which can be transferred by coughing, sneezing or intimate contact. The approach described above for bacterial tonsillitis is easily replicated as a disinfecting (decolonising) treatment for the back of the throat or nostrils and could be utilised in at-risk cohorts. This could be employed in local "circuit-break" decolonisation during epidemic/pandemic outbreaks, such as the current SARS-CoV-2 situation, e.g. for air travel arrivals, nursing homes residents and staff or, indeed, hospital admissions.

Fortunately, the model for such decolonisation approaches is already in existence. The MRSAID® system, developed by Ondine Research Laboratories and Ondine Biomedical Inc. in Seattle/Vancouver has been employed for MRSA decolonisation of the anterior nares (Figure 7a) for several years in Canadian hospitals [64], with over 50,000 elective patients being treated. This has produced a 50 % drop in post-operative MRSA infections – i.e., a decrease without recourse to conventional antibacterial agents, either as pre-operative prophylaxis or post-operative anti-infective therapy. The procedure is rapid and simple, with application of methylene blue to the nostrils, followed by optical fibre-delivered red light illumination [65]. Again the absence of resistance development as a result of the methylene blue approach is underlined.

6.1. New dyes?

The principal dye used in anti-infective procedures in the 21st Century is the phenothiazine (phenothiazinium) derivative methylene blue [66]. This reflects its safe use in various human and animal applications since 1890. In addition, it is the most used photodynamic dye in infection control, mainly due to its almost sole possession as the active in both the oral disinfection and plasma phototreatment roles, as mentioned above.

However, in terms of its photodynamic ability, it is not the most impressive photosensitiser available, even among commercially-available compounds, and various groups have reported improved candidates [67,68]. These have been both phenothiazinium derivatives (commercially available and novel) and belonging to other dye classes, typically the porphyrin and phthalocyanine types [69].

A detailed discussion of ranked candidates ready to replace methylene blue lies outside the scope of the current work and can indeed be found in other reports [70-73]. However, the argument around such a replacement is not straightforward, nor even merely limited to scientific disciplines, given the amount of financial and legislatory input required, as would be the case with any new therapeutic.

Broadly speaking, in order even to achieve increased use even of methylene blue alone there are three main problems to address: the requirement for light activation, the perceived potential for mutation and the staining of tissue.

The requirement for illumination of the applied dye is obviously a departure from conventional antimicrobial therapy where tablet ingestion, solution injection or cream application has been the norm for 80 years. Clearly phototreatment of localised internal infection, e.g. of the lung (see Figure 7b, above), would require medical supervision, both for dye and light targeting, whereas that of a fungal skin lesion could be managed with a commercial metred light source at home, the patient

also applying the dye. In both cases, this is obviously less straightforward and user-friendly than the conventional approach, but with the continuing rises in AMR, the conventional approach increasingly doesn't work. Furthermore, the pipeline of conventional-type antimicrobials is hardly free-flowing. Both of these aspects require positive publicity to support further use.

Similarly, the bad press experienced fairly generally by dyes regarding supposed DNA interference is a result of what might be termed partial information: planar cationic molecules may interact with DNA in isolation (e.g. in buffer in a test tube) or in simple cellular systems *in vitro*, but this is not necessarily the case *in vivo*. In addition, interaction with DNA in an infected individual does not, by definition, result in mutation – as noted, this is demonstrated by the widely used DNA-active quinolone antibacterial class: here, the damage to DNA is limited to bacteria, with no adverse effects on the host. One of the advantages of the rapid treatment associated with the photodynamic approach is that there is insufficient time for complex, mammalian cell uptake of the dyes used. Again, this is rather different to the original dye therapy protocols employed in wound disinfection, for example, but as argued above, there is no evidence to support mutation as a result of this.

Dyes, by traditional definition, are colouring materials. Their use in modern medicine is thus somewhat "off-lable" and skin/tissue colouration is a normal consequence. While this is normally transitory, it is still often considered to be unsightly. However, dye application is proposed here in order to fight conventional drug resistance – either directly against drug-resistant bacteria (for example), or against susceptible organisms in order to conserve valuable conventional drugs. Staining in relation to this might well be considered a small price to pay. This is similar to the view of Garrod in the early 1930s, defending the gynaecological use of brilliant green: *It is objected to on account of its staining propensities; whether stained linen or death from septicaemia is the greater evil is a question which seems to admit of only one answer* [74]. Preparing the patient for the presence of staining is necessary, whether externally as in a skin wound or infected burn, or as a result of internal use which might lead to coloured waste products. Knowledge and explanation of the latter aspect was, and is, key in gaining acceptance for the (conventional) use of methylene blue in childhood malaria in Africa [75].
One way around the staining problem, of course, would be to employ dyes with principal absorbances outside the visible region. However, the introduction of near-infrared dyes would represent a significant amount of testing, given that only one example, indocyanine green, is currently cleared for medical use in humans [76,77].

6.2. How is this approach to be supported?

Due to their demonstrable use in anti-infection in the early part of the 20th Century, the provision of dyes to this end was part of the business of the nascent pharmaceutical industry. This enabled the emergence of recognisable, modern conglomerates in Europe at the time of the major shift from anti-infective dyes to conventional antimicrobial drugs with the release by IG Farben of Prontosil in 1935 and the consequent realisation of sulphonamide action [34].

Clearly, there has been, probably since that time, a distancing of pharmas from their dye industry origins and this makes the concept of modern dyes and photodynamics a difficult sell for those attempting to secure financial and collaborative support. However, a major driver for new drug development is drug resistance, and this is undoubtedly now an enormous problem for this sector - so much so that several pharma houses have left the anti-infectives business. As noted, the most popular alternatives to conventional drugs appear to be biologicals (vaccines, antibodies, phages), but these are relatively expensive to produce and would not, in any case, support the broad-spectrum protection offered by light-activated dyes.

Given that precise light activation is required here for effective antimicrobial action, it is likely that "dye-plus-light-source" products will be needed, which would overlap with the medical device industry. Thus, perhaps hybrids, formed from companies in both sectors, are an answer. This would be in addition to purpose-built start-ups, although these can obviously suffer, as well as gain, from the vagaries of benefactor/venture capital involvement. The lack of interest from long-standing (academic) grant funding bodies means that involvement of the private sector route is probably essential.

As an example, the employment of the photodynamic approach to infected sinuses is mentioned above (Figure 7a). Approximately 3 million cases of acute sinusitis are reported annually in the USA alone [78, 79], accounting for a multi-billion dollar annual market [80]. Particularly in view of the problem of AMR and weak drug pipelines, this should represent a realistic target area for those working on such applications and seeking funding.

While dyes for immediate use would have to be those currently approved and licensed for the clinic, *viz.* methylene blue, toluidine blue and crystal violet, these would require "clean" synthesis and purity documentation, as covered previously. Known, reported and potential improvements on these dyes would require approval and licensing, which would clearly take some time and considerable funding. Consequently, such a process would become more straightforward with the acceptance and increased application of the approaches and dyes already in use. However, again, the highly significant driver of AMR should not be discounted and it is crucial that there is continued – and preferably increased - reporting of *in vitro*/*in vivo* testing and clinical work. The excellent and tireless efforts of Tardivo on infections of the diabetic foot provide an outstanding example of what may be achieved [81], and more work like this is essential in order to gain wider clinical acceptance.

7. Conclusion

It remains paradoxical that the demonstrable light-activated killing of microorganisms using synthetic dyes pre-dates modern antibacterial drugs by some 35 years and yet there is still no broadly-accepted protocol which employs photoantimicrobials in the direct treatment of infection. Bacterial (conventional) drug resistance has been predicted and reported, with increasing regularity, since 1945 and is today a major global cause for concern in affluent societies but the dye-based approach to overcoming such resistance – regardless of mechanism – is not employed. The problem of antimicrobial drug resistance and, more importantly, lack of drug availability also continues to plague less well-funded healthcare concerns in the Developing World. The dyes and technology which might at least partially aid in each of these areas are available; hopefully the leap of faith required for large scale deployment will not be long in coming.

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