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Review

On the 75th anniversary of Prontosil

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ABSTRACT

While much of the credit for the beginning of the "antibiotic era" is given to Fleming, the first clinically available antibacterial agents were the sulphonamides, discovered as by-products of the azo dye, Prontosil. This was given its general release, i.e., outside Germany, in 1935 and rapidly became associated with "miracle" cures, particularly in skin diseases, pneumonia and childbed fever. While the discovery of sulphanilamide as the active agent in Prontosil led to the explosion in "sulpha" drugs other, related, agents such as Marfanil and the thiosemicarbazides were also developed by the Bayer chemists, and knowledge of the breakdown of the azoic bond specifically in the colon has also led to the introduction of drug delivery approaches to that organ.

1. Introduction

The "Antibiotic Era" is often held to have begun with the discovery of penicillin by Alexander Fleming. However, Fleming's original discovery of the anti-staphylococcal action of *Penicillium notatum* "mould juice" occurred in 1928 and the significant clinical introduction of penicillin was not a reality until 1944. In terms of useful antimicrobial drugs, the major contribution during this hiatus was provided by the sulphonamides. This class was derived from a family of azo dyes, the activity of which was first publicised in 1935.

In the early part of the 20th Century the nascent pharmaceutical industry – which itself did not begin to thrive until after the establishment of the sulphonamide class – was closely allied to producers of synthetic dyes, drug research laboratories often screening dyes as lead compounds. Such was the case at the German conglomerate, IG Farben, which had effectively continued the work of Paul Ehrlich in investigating dyes as antimicrobial agents – indeed, one of Ehrlich's students, Roehl, was employed by Bayer (part of IG Farben, along with AGFA, BASF and Hoechst) and developed some of the early antimalarials, work which eventually realised the globally important chloroquine [1].

Gerhard Domagk was not one of Ehrlich's students, but his experiences as a medic during the First World War provided him with enormous drive to fight infectious disease. Domagk began his search for anti-infectives, again working for Bayer, in the late 1920s.

Plainly, screening dyes for antimicrobial use requires a constant supply of test compounds. Fortunately for Domagk, as for Roehl, the research chemists tasked with the supply were highly talented. Werner Schulemann, Friedrich Mietzsch, Hans Mauss and Joseph Klarer were involved in much of Bayer's better drug discovery chemistry, Mietzsch and Klarer being principally involved in providing those azo dyes which culminated in Domagk's success.

In the 1930s, azobenzene derivatives (Aryl–N=N–Aryl) were well established as dyestuffs, mainly for the textile industry, which was still a thriving concern at that time. However, despite the belief that drug action must be related to dyeing facility, the target cells becoming the substrate, the colour of the compounds synthesised for Domagk's experimentation was not of major concern. However, because of the improved wash- and lightfastness of textile azo dyes containing it, the presence of the sulphonamido group ($-SO_2N<$) was identified as an important indicator of potential drug activity from an early stage. Indeed, Mietzsch had a commendably modern approach to his particular task of antibacterial drug discovery, which was effectively that of molecular design: "..the right substituents in the right positions on the azo group" [2].

For the various chemists at Bayer/IG Farben involved in the search for improved therapeutics based on dyes, the lead compound in antimalarial research was the phenothiazine dye, methylene blue. This was converted into a more effective agent by altering one of the dimethylamino auxochromes to one having basic character. This was achieved by connecting a second amino residue to the auxochromic group by way of an inert alkylene linker. The resulting aminoalkylamino side chain, and its later derivatives, was attached to several different dye classes, including

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phenoxazines, triarylmethanes and acridines, from which the widely used acridine, Atabrine (Mepacrine, Quinacrine), was produced [3].

Azo dyes (Fig. 1) had, in fact, already been suggested and trialled *in vitro* as antiseptic agents as early as 1913. Eisenberg reported excellent results for the simple dye chrysoidine [4], as did Ostromislensky for pyridium and serenium [5] The latter two were still in use as urinary antiseptics in the 1930s [6], and pyridium remains a commonly prescribed preparation [7]. However, such results were not observed in Bayer's animal testing [3].

Perhaps not surprisingly, then, the initial azo-compound given to Domagk for testing, KL695 (KL = Klarer), contained both the sulphonamide and aminoalkylamino residues (Fig. 2). The dye KL730, which became known as Prontosil, was a simple analogue of the azo dye chrysoidine [8], having the straightforward addition of a sulphonamide group (Fig. 2).

While it is difficult, in hindsight, to be impressed by the antibacterial effects of these two simple compounds, this remains one of the major scientific breakthroughs of the 20th Century. It should not be forgotten that Domagk and his associates were working in an era where there was, as yet, little knowledge of drug metabolism and it is probable that they had little regard for the reactivity of the azoic linkage in mammalian systems. However, the activity of these compounds, and others, led to the patenting of several series, starting at the end of 1931, with Mietzsch and Klarer given as inventors. It should also be noted that Domagk's award of the Nobel Prize for Medicine in 1939 (received in 1947) was made to him alone, and not shared with the drugs' inventors.

As mentioned, the discovery of the antibacterial activity of these azo dye sulphonamides was made around 1930–31, as the result of an industrial medicinal chemistry project. Given the commercial nature of the work, the data were held in-house pending further investigations. However, the results were unusual in that there was a lack of antibacterial activity *in vitro*, the dyes being successful in Domagk's model for human septicaemia, mouse peritonitis induced by a clinical isolate of *Streptococcus pyogenes*. The extravagance of candidate screening by duplicating *in vitro* tests in animals would be difficult to justify in the 21st Century, but it is fortunate that it occurred in this case. Domagk's first experiment, using twenty-six infected mice, resulted in the twelve which received Prontosil surviving, whereas the remaining controls died.

KL730, initially known at Bayer as *Streptozon*, underwent many months of animal testing. It was presented and discussed at scientific meetings within Germany, and several preliminary literature reports of its activity appeared. One example concerned the cure of a 10-month old baby boy, dying from staphylococcal septicaemia [9], and cures were also claimed for other typically staphylococcal or streptococcal diseases, such as erysipelas, scarlet fever and puerperal fever. Again, it is difficult, even in these days of hospital superbugs, to appreciate the impact of these findings. However, the fact that such diseases are relatively unknown today is due, in part, to the initial inroads made by the sulphonamides.

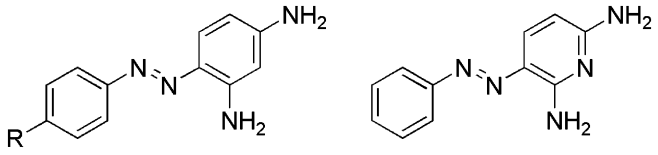

Fig. 1. Early, *in vitro*-active antiseptic azo dyes.

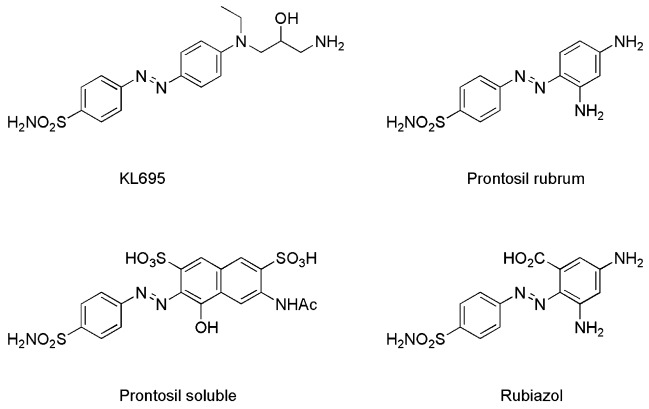

Fig. 2. Initial azo dyes having significant antibacterial action *in vivo*.

In the 1930s, despite considerable advances in diet and social conditions, puerperal, or childbed, fever was a significant threat, and one of the principal, and successful, initial trials of Prontosil (the name Streptozon had been dropped by 1935) against this disease was supervised by the English clinician Leonard Colebrook at Queen Charlotte's Maternity Hospital in London [10]. There were, of course, more famous incidences of cures – for example, Domagk's daughter Hildegard [11] and Franklin Delano Roosevelt Jr., one of the sons of the then US President, both from streptococcal septicaemia [12]. The proper, clinical introduction of Prontosil thus occurred in 1935, the same year as Domagk's landmark publication, understatedly entitled: A contribution to the chemotherapy of bacterial infections [13], and the presentation of the Prontosil work to the Royal Society of Medicine by Heinrich Horlein, Medical Director of IG Farben [3].

Prontosil (also known as *Prontosil rubrum* due to its dark red colour) was initially administered orally as a hydrochloride salt, and later as the free base as this was less staining. However, both preparations were relatively insoluble in water, and recourse was made to the library of prepared, patented compounds for a more soluble active derivative for use in injectable formulations. Prontosil soluble (Fig. 2, originally *Streptozon S*) was produced by the reaction of diazotised sulphanilamide with 2-acetamino-8-hydroxynaphthalene-3,6-disulphonic acid. This proved to be eminently suitable for use in aqueous media and allowed far more rapid administration of the drug in cases of serious disease, often in conjunction with oral Prontosil.

As noted, many azo derivatives were produced during the drug discovery phase by Mietzsch and Klarer – indeed Horlein claimed over one thousand derivatives by 1936 [3]. The molecular requirement for the relative 1,4-disubstitution pattern of the sulphonamide and azo moieties was established by this time, but it was the French team under Fourneau which made the quantum leap in suggesting that it was a dye metabolite common to all the successful derivatives which was, in fact, the active agent [14]. Sulphanilamide is formed via the reduction of the azoic bond in each case (Fig. 3).

In fact, the canine metabolism of an azo dye, Orange I, notably covering both the degradation of the –N=N– moiety and the identification of sulphanilic acid in the urine, had been reported as early as 1911 [15], while the presence of sulphanilamide in human urine following Prontosil administration was first described by Fuller in 1937 [16].

This metabolic activation of Prontosil and its congeners explained the lack of activity of the compounds during *in vitro* screening, and it was a logical step for the French team to test the precursor sulphanilamide itself for antibacterial efficacy. The resulting high activity of

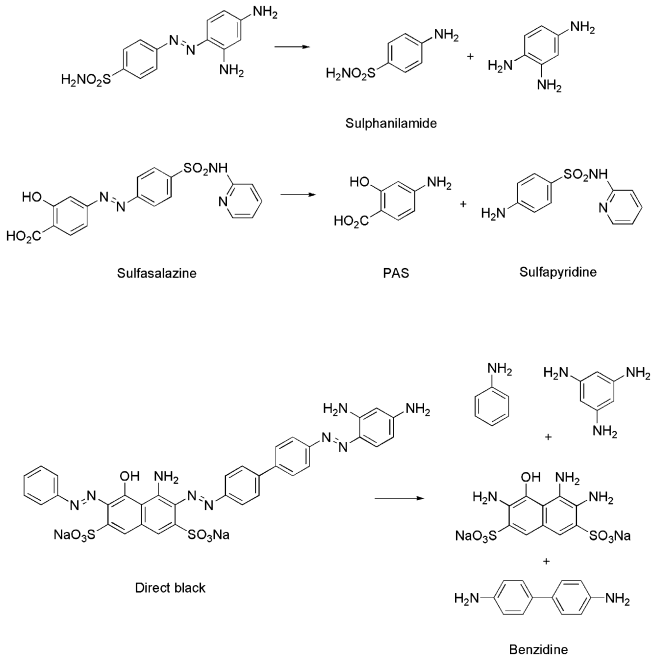

Fig. 3. Reduction of the azoic bond to provide therapeutic and/or harmful agents.

sulphanilamide represented a considerable problem to IG Farben: although it was immediately marketed as *Prontosil album*, no patent could be granted, as this was a known compound, ironically first patented by Bayer in 1912 [17]. On the face of it, all of the patents covering the azosulphonamides were now of little worth. However, parallel work on benzylamino and other derivatives led to the introduction of Marfanil and the Ulirons (Fig. 4), of great value against anaerobic bacteria such as those implicated in gas gangrene, obviously a particular problem subsequently for the *Wehrmacht*.

The realisation of the requisite sulphanilamide skeleton for antibacterial activity, of course, led to a considerable increase in the synthesis of novel derivative series, this representing the initial rush of "me too" compounds. The first of these to become clinically significant was sulfapyridine (Fig. 3), or M&B693, later to be involved in another famous cure – this time of Winston Churchill's pneumonia following his gruelling series of meetings in the Middle East in December 1943 [18]. Many other examples followed, one of which, sulfamethoxazole, is still in front-line clinical use.

2. Pharmacology

The establishment of sulphanilamide as the active part of Prontosil and its congeners led, in turn, to investigations of its mode of action, and this was discovered to be the inhibition of activity (usually referred to as *antagonism*) of *para*-aminobenzoic acid (pABA) by Woods and Fildes in 1940 [19]. The structural similarity of the two compounds is striking (Fig. 4), and this was the first instance of the molecular basis of drug action being clearly indicated – sulphanilamide action can, of course, be reversed by the addition of pABA.

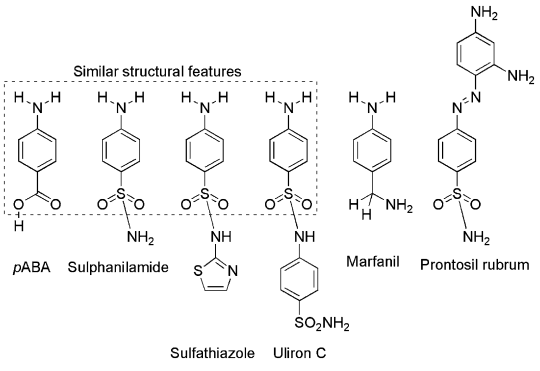

Fig. 4. Structural similarities allowing inhibition of pABA.

It should be pointed out that the action of sulphanilamide and its congeners against pABA is focussed on an bacterial enzyme, dihydropteroate synthase, which is essential in the synthesis of folic acid [20]. However, the structural similarity to pABA shown for sulphanilamide, Uliron C and sulphathiazole in Fig. 4 is not shared by either Marfanil or Prontosil. While it was understood by the late 1930s that the Prontosil drugs were, in fact, sulphonamide pro-drugs, Marfanil and its derivatives do not exhibit sufficient commonality with pABA, and do not antagonise this. The far greater activity of these drugs against *Clostridium* spp., e.g. in gangrene, is explained by a different mode of action [21], possibly similar to the cationic disinfectants.

With hindsight of the pro-drug nature of the Prontosil and the essential molecular skeleton required for sulphonamide antibacterial action, many of the findings from the early work carried out at Bayer may seem routine, for example, the presence of acidic groups in the sulphonamide-containing aryl moiety led to lowered activity [22], since this ring has no effect on sulphonamide action. Rubiazol (Fig. 2), the version of Prontosil marketed in France by Rhône-Poulenc – there was no contemporary patent-honouring arrangement with Germany – contained a carboxylic acid residue in the 3′-position, with no apparent loss in activity. However, why should derivatives having *N*-alkyl or *N*,*N*-dialkyl substitution of the sulphonamide group have provided active antibacterials [23], while *N*-(2-hydroxyethyl) analogues did not [22]?

The patent literature protecting the great work of Mietzsch and Klarer demonstrates clearly how inventive, forward-thinking and industrious they were, and firmly underlines their claims for inclusion alongside Domagk when the accolades began to arrive. However, given Domagk's problems with Nazi officialdom, including being imprisoned by the Gestapo, such accolades were somewhat double-edged. It is interesting, nevertheless, to contrast the low degree of recognition of the two Bayer chemists with that of Fleming, whose part in the penicillin story was relatively minor [24]. The chemists' battery of azo dyes was certainly considerable, ranging from simple benzenoid derivatives like Prontosil itself, through naphthalene analogues, such as Prontosil Soluble, to heterocyclic derivatives, mainly based on pyridine and quinoline [22].

From a 21st Century standpoint, with the advantage of decades of drug discovery experience, it may seem strange that a compound such as Prontosil rubrum could have been released for human use. The breakdown of the azoic linkage necessary for the release of the active sulphanilamide also produces 1,2,4-triaminobenzene (Fig. 3), which is both a primary arylamine and a *p*-phenylenediamine derivative and thus readily oxidised to a quinoneimine Michael acceptor. Clearly the potential for toxicity of this by-product would preclude its use today, and it is a known carcinogen [25]. The fact that Prontosil rubrum remained in everyday use in Germany throughout World War Two, despite the establishment of sulphanilamide as the active agent is puzzling, however, particularly since sulphanilamide itself (as Prontosil album or Prontalbin) was also in great demand.

3. Discoveries following Prontosil

As noted, the discovery of Prontosil, momentous as it was, also led to the discovery of other, improved drugs based on the sulphonamide group. As well as the sulphonamide class itself, this

includes important antimycobacterials such as the sulphone and thiosemicarbazide classes, for Hansen's disease and tuberculosis respectively. Both of these compound types were also synthesised and screened by Bayer, and it is unlikely that its discovery would have been as rapid (mid-1940s) had it not been for the initial Prontosil breakthrough.

During the early 1940s it was also found that some sulphonamide examples could also promote the release of insulin from beta cells in the pancreas, leading to another drug use in later years as oral hypoglycaemic agents [26]. It is possible that more modern sulphonamide derivatives, used in this way, might also be useful as antimicrobial agents, following a similar paradigm to that which has shown that neuroleptic phenothiazines, derived from the antimicrobial methylene blue, are useful antibacterial agents via their concentration in macrophages [27].

While it may be supposed that the discovery of the active nature of sulphanilamide sounded the death knell for the use of azo dyes in therapy, this has not been the case. Rather, knowledge of the metabolic breakdown of the azoic linkage has been used in drug delivery. Since the breakdown occurs in the colon it supplies a method of targeting, for example in the bowel disorders Crohn's disease and ulcerative colitis [28]. Here, sulfasalazine is often employed in therapy, being broken down into its constituent parts *para*-aminosalicylic acid and sulfapyridine (Fig. 3). The azo linkage has also been incorporated into hydrogels, to be employed in the colonic drug delivery, for example, of antitumour therapeutics [29]. Prontosil soluble has also recently been reported as an excellent inhibitor of β-carbonic anhydrase, a novel drug target obtained from the elucidation of the genome of *Mycobacterium tuberculosis* [30]. In addition, the metal complexing capabilities of salicylic acid derivatives have led to the development of antimicrobial azo dyes utilising metal ion toxicity to provide the cell killing effect [31]. Such activity can, of course, be applied quite logically to the antimicrobial protection of textiles, and this has been demonstrated using the simple expedient of quaternary ammonium inclusion within the azo dye molecule [32]. Obviously, this is an area where the colour of the active is not considered to be a negative aspect, and given the degree to which the contemporary hospital environment is culpable in infectious disease transmission, this offers a promising approach to textiles used in the healthcare milieu.

Additionally, knowledge concerning the breakdown of the azoic linkage is of great utility in protecting those exposed in various ways to azo dyes, for example in the prediction of likely metabolites (Fig. 3). Conversely, the bacterial degradation of Prontosil, associated with enzymes such as the azoreductase group, has found use in the decontamination of wastewater in the dye industry itself [33].

4. Conclusion

Drug development is an evolutionary process, usually beginning from a lead compound. In the case of the Bayer dye chemists in the late 1920s, two chemical groupings were used rather than a single molecule. Both the basic side chain and the sulphonamide group were present in what became a chemical lead, KL695, and the Prontosil compounds were developed from this. However, the sulphonamide moiety was thought to be necessary as a result of improved dye fastness – not entirely related to drug action – and the basic side chain was part of the non-active region of molecules which were eventually realised to be pro-drugs. Given the lack of knowledge of metabolic activation, there was clearly a degree of fortune at work in the project. However, it should be remembered that Mietzsch and Klarer, whose inventiveness ultimately led to success, were always aiming at the production of a small antibacterial molecule. This is some way from the serendipitous discovery made by Alexander Fleming.

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