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Dihydrofolate reductase inhibitors as antibacterial agents

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ABSTRACT

Although only a few DHFR inhibitors have progressed as antibiotics to the market there is much renewed interest in the discovery and development of new generation DHFR inhibitors as antibacterial agents. This article describes the success in exploiting DHFR as a drugable target as exemplified by trimethoprim (TMP) and the development of several new diaminopyrimidines. Iclaprim, a recent example of a novel diaminopyrimidine currently in Phase III clinical trials, is also described together with several examples of anti-DHFR antibacterial compounds in pre-clinical development.

1. Background

Both prokaryotic and eukaryotic cells require reduced folate cofactors for the biosynthesis of a diverse range of cellular components. Although most bacteria and plants produce these folate cofactors by de novo biosynthesis, some bacteria and mammalian cells use preformed folates and exhibit salvage pathways for reduced folates and pyrimidines. As shown in Fig. 1, six enyzmes are involved in the biosynthesis of tetrahydrofolic acid (THF), starting from guanosine triphosphate, including dihydrofolate reductase (DHFR, E.C.1.5.1.3). Tetrahydrofolate cofactors are essential for the synthesis of purines, some amino acids and thymidine.

DHFR plays a central role in maintenance of cellular pools of THF and its derivatives, including methylation of dUMP to dTMP, and in cell growth and proliferation. DHFR is the sole source of THF, and as such is an Achilles' heel of rapidly proliferating cells. The enzyme is the target of several important anticancer and antibacterial drugs. Indeed, the efforts to identify the mechanism of action of the anticancer drug methotrexate lead to the discovery of DHFR [1]. There are evident differences between the eukaryotic and prokaryotic DHFR's which have lead to the discovery of the antibacterial agent trimethoprim (TMP). TMP binds bacterial DHFRs 10^5 times tighter than it does to vertebrate DHFRs [2]. By virtue of their mechanism of action antibacterial DHFR inhibitors exert their activity through blocking synthesis of DNA, RNA and proteins, thereby arresting cell growth.

DHFR is widely conserved and essential in bacterial pathogens and the clinical success of TMP demonstrates that this enzyme is a useful target for the discovery of novel antibacterial agents.

2. Exploitation of DHFR genomics and DHFR biochemistry in the design of new antibacterials

All living cells need tetrahydrofolate cofactors for the synthesis of purines, some amino acids and especially of thymidine and their biosynthesis from GTP is well described ([3–5], http://www.genome.ad.jp/kegg/pathway/map/map00790.html). DHFR is perhaps the best studied enzyme in the folate pathway. Significant structural information is available with over 100 different DHFR crystal structures available (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=Pager&DB=structure). Moreover, 3D-structures of DHFR have been used to understand inhibitor binding, enzyme:inhibitor:cofactor complexes and differences between bacterial and mammalian DHFR. For example, the atomic structure of human DHFR complexed with NADPH and two lipophilic antifolates, the 3D-structure of the *Mycobacterium tuberculosis*

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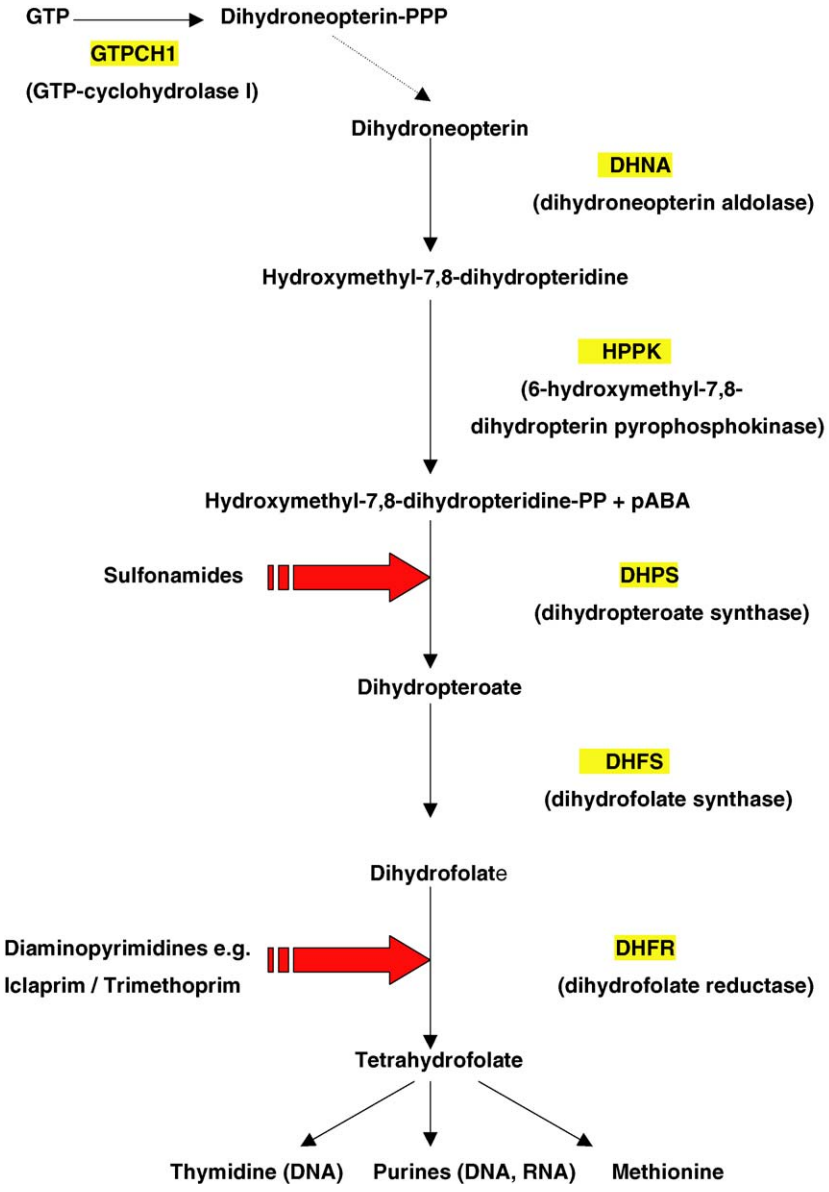
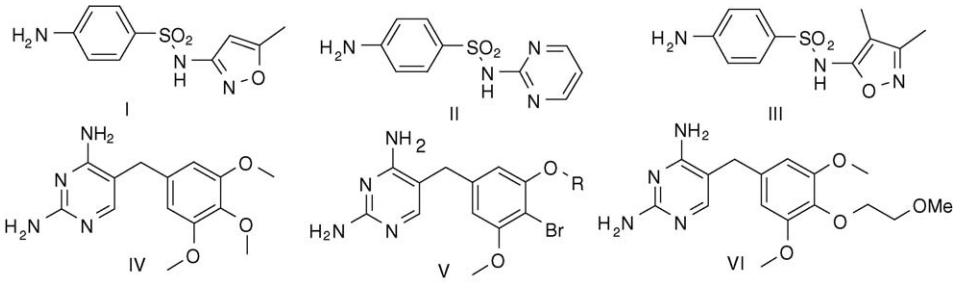


Fig. 1 – Biosynthetic pathway of tetrahydrofolic acid in bacteria.

DHFR complexed with several inhibitors, and many others including a variety of DHFRs from clinically important Gram-positive and Gram-negative bacterial pathogens are now available [6]. Such information can be exploited for directed design of new and selective DHFR inhibitors. The plethora of information on DHFR makes it an important drugable target for the development of novel antibacterials. Moreover, information on the mechanism of action and mechanisms of resistance leaves the door wide open for research and discovery of new agents that exert their antibacterial action through specific and selective inhibition of DHFR.

3. DHFR antibacterial agents in clinical use

TMP (see Scheme 1), a 5-substituted-2,4-diaminopyrimidine antibacterial agent, is a selective inhibitor of bacterial DHFRs. TMP was initially used as monotherapy for the treatment of certain forms of urinary tract infections in the mid-1970s. In terms of the antibacterial properties of TMP, the agent exhibits a broad spectrum of activity against a wide variety of Gram-positive bacterial pathogens in vitro comprising *Staphylococcus aureus*, both methicillin-sensitive (MSSA) and methicillin-resistant (MRSA), *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Streptococcus viridans*, *Enterococcus faecalis* and Gram-negative bacteria including *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus* spp., *Salmonella* spp. and *Haemophilus influenzae* are sensitive to TMP. However, *Pseudomonas aeruginosa*, *Acinetobacter*, *Moraxella*, *Neisseria*, *Brucella*, *Campylobacter* spp., *Nocardia*, *Actinomyces*, mycobacteria, *Bacteroides*, *Clostridium* spp. and *Chlamydia pneumoniae* are all less sensitive or resistant to the action of TMP or other benzyl-pyrimidines [7]. In addition to the broad spectrum of activity, TMP also shows a bactericidal action albeit at multiples of MIC concentrations. Approximately 60% of the parent compound is excreted in the urine



Scheme 1 – Sulfamethoxazole (SMX, I), sulfadiazine (II), sulfisoxazole (III), trimethoprim (TMP, IV), brodimoprim (V, R=CH_3), tetroxoprim (VI).

and the high concentration achieved results in an excellent efficacy in urinary tract infections. TMP has low or no acute toxicity and a good safety profile although at high doses nausea is observed [7].

Resistance to TMP in bacteria can be caused by a variety of mechanisms. Point mutations, which affect binding of the inhibitor, have been described in several Gram-positive bacteria. By contrast, resistant bypass enzymes, spread by mobile genetic elements, are the main cause of resistance in Gram-negative bacteria [8]. Almost 20 different TMP-resistant DHFR bypass enzymes have been found in Gram-negative bacteria and a detailed discussion of their characteristics, their genetic location and epidemiology is beyond the scope of this article. For example, resistance to TMP in *Streptococcus pneumoniae* is due to a single mutational amino acid change in the *Streptococcus pneumoniae* DHFR, from isoleucine to leucine at position 100 [9,10]. Similarly, a single amino acid substitution from phenylalanine to tryptophan at position 98 was found to be responsible for low to medium level TMP resistance in *S. aureus* [5]. X-ray crystallography with the ternary complex of the F98Y DHFR with folate-NADPH showed that the mutation resulted in a loss of a hydrogen bond between the 4-amino group of TMP and the carbonyl oxygen of Leu5. This mechanism is predominant in both transferable plasmid-coded and non-transferable chromosomally encoded resistance [11].

Although TMP has been used clinically as a monotherapy, it is the combination drug (TMP and sulfamethoxazole, a sulfonamide; Scheme 1) that has been widely used as an antibacterial agent for over three decades. Sulfamethoxazole acts on an additional enzyme in the folate pathway, namely dihydropteroate synthase (DHPS, E.C.2.5.1.15) and the combined action of TMP and sulfamethoxazole (e.g. in cotrimoxazole) results in synergistic action. Such synergistic combinations of activity have also been observed for TMP in combination with other sulfonamides including sulfadiazine and sulfisoxazole (Scheme 1). Cotrimoxazole is generic and still widely used. Its main use is for the treatment of uncomplicated forms of urinary tract infections as well as prophylaxis and treatment of *Pneumocystis carinii* pneumonia in HIV patients.

Milestones in the clinical use of antimicrobial DHFR inhibitors were the introduction of the combination of TMP with sulfamethoxazole in 1968, the introduction of TMP alone in Finland in 1972 and the launch of a new combination of tetroxoprim [12] with sulfadiazine in 1979.

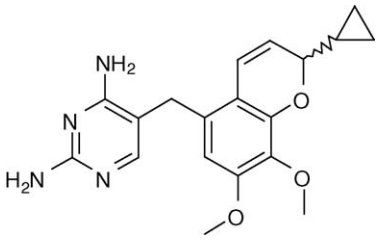
4. Recent diaminopyrimidines

Intensive research, primarily performed at Roche, lead to the synthesis and characterization of various novel diaminopyrimidine compounds. Although the vast majority of these diaminopyrimidines possessed similar though not superior microbiological activity as compared with TMP, several did possess properties that were considered to be potentially advantageous. One such example of an improved diaminopyrimidine is brodimoprim (Scheme 1). Although the antibacterial and toxicological properties of brodimoprim are similar to those of TMP, its pharmacokinetic behaviour is distinctly different. In contrast to TMP, brodimoprim has a long elimination half life of 32–35 h, which permitted once daily dosing as compared with the bi-daily dosing for TMP and co-trimoxazole. Unlike TMP, brodimoprim is mainly excreted through the bile and faeces. Brodimoprim shows a high volume of distribution and good tissue penetration [13]. Taken together, these characteristics lead to the development of brodimoprim for the once daily treatment of respiratory tract infections [14,15]. Brodimoprim was initially marketed for the treatment of respiratory tract infections in Italy in 1993, but did not find wide acceptance and was withdrawn from the market in 2000.

Epiroprim is a diaminopyrimidine antibiotic that is more lipophilic than TMP. Epiroprim possesses attractive properties for the treatment of Gram-positive infections or infections caused by several opportunistic protozoa. Of particular note is the potent activity against *Mycobacterium ulcerans* and observed synergism when combined with dapsone [16,17]. Epiroprim was also synergistic with dapsone against *Mycobacterium leprae* in vivo [18]. To the best of our knowledge there is no development work currently being pursued with epiroprim.

5. Novel DHFR inhibitors in clinical development

Based on the demonstrated clinical efficacy and excellent safety profile of TMP, companies and academic institutions have continued to drive forwards drug discovery programs aimed at discovering novel diaminopyrimidines. A prime example of these efforts is the novel diaminopyrimidine iclaprim (Scheme 2) which shows marked differences in activity as compared with TMP. Iclaprim has successfully



Scheme 2 – Iclaprim (AR-100, MW = 354).

completed Phase II clinical trials and is currently in Phase III clinical development.

Based on the finding that point mutations in the DHFR enzyme were mainly responsible for resistance to TMP and that in *S. aureus* the enzyme could be efficiently inhibited by methotrexate [19], Roche initiated a program to find improved inhibitors of DHFR. Structural information and molecular modeling approaches lead to the synthesis of diaminopyrimidines that were more active than TMP particularly against Gram-positive pathogens. Importantly, these compounds gained activity on TMP-resistant strains and in addition showed potent activity against methicillin- and vancomycin-sensitive and -resistant strains. One compound emanating from this rational drug design program is iclaprim (formerly AR-100 and Ro 48-2622). Although initially discovered at Roche, iclaprim has been in-licensed by Arpida which has developed this compound through pre-clinical and clinical trials.

Iclaprim is a racemic mixture composed of two enantiomers for which a synthesis for one of the enantiomers is reported by Wipf and Weiner [20]. Both enantiomers have been shown to be equipotent against various bacterial DHFR enzymes and in terms of their antibacterial activities [21]. Iclaprim, like TMP, specifically and selectively inhibits Gram-positive and Gram-negative DHFRs at sub-micromolar concentrations with little or no inhibition of the human enzyme at over five orders of magnitude higher concentrations. One of the major differentiating factors between iclaprim and TMP is that iclaprim also exhibits potent activity against various TMP-resistant DHFRs including DHFRs from TMP-resistant *S. aureus* and *Streptococcus pneumoniae* [21] and is more potent than TMP against DHFR from *P. carinii* [22]. The modes of action of iclaprim and TMP have been studied in intact bacterial cells using radioactive precursors to detect changes in the major macromolecular syntheses of DNA, RNA, protein and cell wall [21]. These studies show that both iclaprim and TMP preferentially inhibit the synthesis of DNA and RNA synthesis with little or no effect on the other macromolecular syntheses observed as would be predicted for selective inhibitors of bacterial DHFR [22].

Microbiological studies employing large panels of bacterial pathogens from various locations worldwide including the United States and Europe have demonstrated that iclaprim exhibits a broad spectrum of activity. As compared with the activity of TMP, iclaprim shows a markedly more potent activity against the major Gram-positive pathogens, particularly staphylococci and streptococci and including many strains that are resistant to TMP. Such improved microbiological activity is in agreement with the inhibitory potency of iclaprim on both the TMP-sensitive and -resistant DHFRs and represents an important differentiating factor from TMP. Moreover, iclaprim has also been reported to exhibit potent activity against Gram-positive pathogens resistant not only to TMP, but also to other clinically used antibiotics including methicillin and oxacillin (e.g. methicillin-resistant *S. aureus* or MRSA), macrolides, quinolones and glycopeptides (including activity against VISA and VRSA) [22].

Iclaprim shows a broad spectrum of activity and like TMP shows good activity against major Gram-negative pathogens, including *Enterobacter*, *Salmonella*, *L. pneumophila* and *H. influenzae* [22]. Moreover, unlike TMP, iclaprim also exhibits useful activity against *Neisseria*, *M. catarrhalis* [22] and *C. pneumoniae* [23].

In contrast to TMP, which exhibits bactericidal activity at multiples of the MIC, iclaprim is rapidly bactericidal on various pathogens at concentrations close to its MIC. For example, iclaprim is rapidly bactericidal against Gram-positive pathogens, including TMP-resistant MRSA, vancomycin-intermediate *S. aureus* (VISA), TMP-resistant *Streptococcus pneumoniae* and vancomycin-resistant enterococci with 99.9% reductions in bacterial loads occurring within 6 h post-treatment [24]. In addition to its bactericidal activity, iclaprim also exhibits a significant post-antibiotic effect of up to several hours [24].

Consistent with its mechanism of action, iclaprim exhibits significant in vitro synergy with sulfonamides such as sulfamethoxazole and sulfadiazine [25]. However, based on its potent and bactericidal activity, it is currently in development as monotherapy. Very importantly, no antagonism has been observed for combinations of iclaprim with penicillins, cephalosporins, penems, macrolides, quinolones and glycopeptides, amongst others [25].

Since the introduction of TMP in clinical use there has been some development of resistance in bacteria resulting in reduced susceptibility. However, it is important to note that resistance to TMP has developed in Gram-positives during three decades of extensive and possible misuse of the drug. More recent data show that TMP resistant rates are now significantly lower at circa 4% in *S. aureus* [26]. In general, spontaneous resistance rates of 10^{-9} are observed with TMP in *S. aureus* and experiments designed to determine the potential to induce resistance shows that already after less than five passages at sub-inhibitory concentrations of the antibiotic, resistance emerges due to point mutation in the DHFR gene (Phe98Tyr) [27].

Studies conducted with either a TMP-sensitive or TMP-resistant *S. aureus* showed that the propensity of resistance development with iclaprim is very low. Indeed, resistant colonies could not be isolated during single-step or spontaneous mutation studies employing these *S. aureus* strains with frequencies of resistance to iclaprim reported as being below 10^{-10}. Furthermore, UV mutagenesis studies employing *E. coli* have also shown that colonies resistant to iclaprim are not detected with frequency of resistance in *E. coli* being once again below 10^{-10} [27]. Similarly, in induction of resistance experiments with TMP-sensitive *S. aureus* little change in iclaprim sensitivity is observed even after 15 passages at a sub-inhibitory antibiotic concentration as compared to the development of high level resistance with TMP after less than five

passages [27]. A similar induction study was also performed with a TMP-resistant MRSA strain whereby little change in sensitivity to iclaprim is observed even after 15 passages of the drug [27].

The interaction of TMP to the resistant *S. aureus* DHFR (mutation of Phe98 to Tyr98) is represented as a sketch based on the reference by Dale et al. [11]. When compared with the wild-type enzyme, the hydrogen bridges of the 4-amino group of TMP are disrupted due to the turn of the Phe92 carbonyl and the occupation of the Leu5 carbonyl by Tyr98 (Fig. 2a). Fig. 2b represents the active site from an X-ray structure of a resistant *S. aureus* DHFR co-crystallized with iclaprim. A potential hypothesis for the increased binding of iclaprim and its ability to inhibit the trimethoprim-resistant enzyme may be based on the new lipophilic interactions of the cyclopropane ring with Leu54 and Ile50 which compensate for the binding losses in the diaminopyrimidine region. These differences in the binding ability of the two drugs might be a possible explanation for the differences in the inhibitory activity of iclaprim when compared with TMP [22]. Superimposition of the diaminopyrimidine rings of both enantiomers aligns part of the cyclopropyl rings close to a similar space suggesting a comparable binding and equipotent activity (data not published).

In addition to the activity of iclaprim in vitro the drug has also been documented as showing efficacy in different animal models of infection [22]. These include septicaemia, peritonitis and pneumonia. These studies show that iclaprim is efficacious following intravenous, subcutaneous and oral administration.

Iclaprim is currently in development as an intravenous therapy for serious complicated skin and skin structure infections (cSSSI) for which patients require to be hospitalized

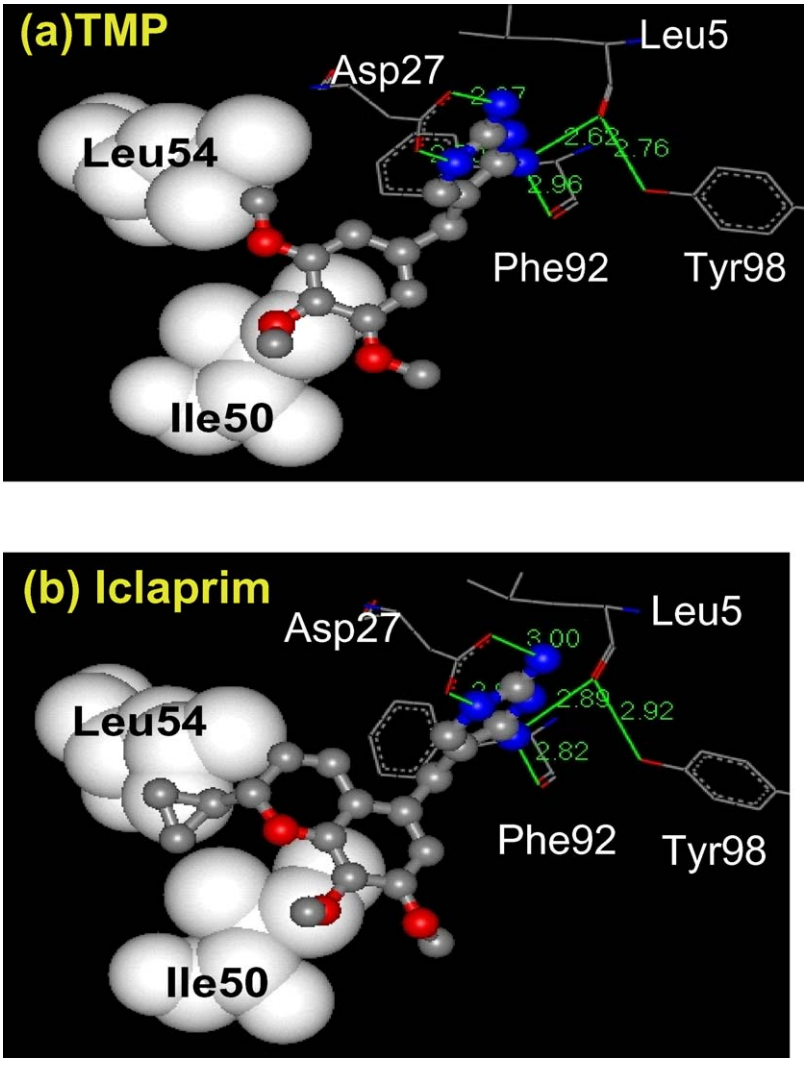


Fig. 2 – Comparisons of the binding of trimethoprim and iclaprim to *Staphylococcus aureus* mutated DHFR. (a) Interactions of trimethoprim with *S. aureus* TMP-resistant DHFR (mutation of Phe98 to Tyr98) based on Dale et al. [11] and (b) active site from an X-ray structure of *S. aureus* TMP-resistant DHFR co-crystallized with iclaprim. Both TMP and iclaprim possess a strong hydrogen bonding with Asp27 with hydrogen binding to the CO of Phe92 stronger for iclaprim as compared with TMP (confirmed by NMR studies). Notably the hydrogen bond of Tyr98 to CO of Leu5 reduces the strength of the hydrogen bond for both TMP and iclaprim. However, unlike TMP, iclaprim possesses the additional interaction with the cyclopropane ring with Ile50 and Leu54 which compensates for the losses. Notably, TMP does not possess additional interactions.

due to the severity of the disease(s). Data from a recent investigator-blinded, multicenter study [28] on 92 patients with cSSSI showed clinical cure rates of 92.9% and 90.3% for iclaprim as a 0.8 mg/kg bid and 1.6 mg/kg bid, respectively. By comparison the standard of care therapy vancomycin exhibited a clinical cure rate of 92.9%. In addition to the high clinical cure rates, eradication rates of Gram-positive pathogens were 90% and 80% for 0.8 and 1.6 mg/kg iclaprim, respectively and were higher than the eradication rate of vancomycin of 70%. The study drugs were well tolerated with no severe drug related adverse events observed. Iclaprim is currently in Phase III trials of cSSSI and was recently granted fast track status by the USA FDA.

In addition to the clinical efficacy and safety of iclaprim in cSSSI a recent Phase I study showed that in healthy subjects the kinetics of iclaprim in the three compartments of the respiratory tree paralleled those measured in plasma. Importantly, the concentrations of iclaprim in the epithelial lining fluid (ELF) and alveolar macrophages (AM) were about 20 and 40 times higher than in plasma, respectively [29]. This study strongly suggests that iclaprim could also be efficacious in the treatment of pneumonia, for example due to staphylococci, pneumococci and other respiratory tract pathogens.

Iclaprim is also orally bioavailable and is currently in Phase I clinical trials as an oral formulation [30]. The oral administration would allow intravenous to oral switch therapy. Such a switch therapy could be used not only following intravenous iclaprim administration but also following administration of other intravenous drugs, e.g. vancomycin. Currently there are only limited options for intravenous to oral switch therapy, particularly for MRSA. If successful, the clinical use of oral iclaprim could, therefore, confer upon iclaprim significant advantages over other antibacterial agents especially regards to the potential in reducing hospital stay and associated costs.

6. Other DHFR antibacterial agents in discovery

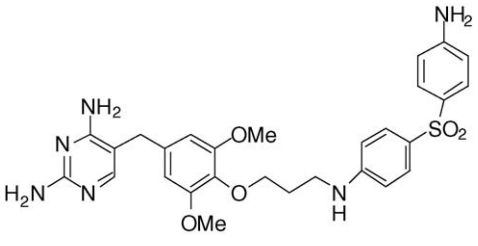
In order to provide advantages and to be significantly differentiated from TMP, new DHFR antibacterial agents require to possess additional properties as compared with TMP. Examples of additional or novel properties of such new agents could be envisaged as including the following characteristics: improving the binding properties against DHFR enzymes, especially against DHFR enzymes that resist the action of TMP and gaining antibacterial activity against TMP resistant strains both in the in vitro and in vivo settings. New compounds could either possess a very broad spectrum of activity or more focused spectra of activity, for example, activity against *Toxoplasma gondii*, *P. carinii* and *Mycobacterium avium* [31–33].

Indeed, although iclaprim is currently the only DHFR antibacterial inhibitor in clinical development, the company Arpida is continuing to expand its efforts in the discovery of novel inhibitors of bacterial DHFR. In addition to iclaprim, the company has discovered an additional novel antibacterial compound, AR-709, which also exerts its antibacterial activity through inhibition of bacterial DHFR. This drug candidate is a broad-spectrum antibiotic that targets pathogens which cause serious respiratory tract infections (''RTI'') in the community setting, including pathogens resistant to several commonly used antibiotics. AR-709 stems directly from a rational drug design approach and is at present in late pre-clinical development [30].

An additional important area of DHFR drug discovery is in the area of targeting new antibacterials for highly specific indications such as for the treatment of *M. tuberculosis*. *M. tuberculosis*, especially multiresistant strains, poses emerging threats to public health in many countries and new drugs are urgently needed. No DHFR inhibitor is currently used for tuberculosis treatment. Epiroprim has recently been evaluated against various *M. tuberculosis* strains [18,31] and found to exhibit weak activity. The drug was synergistic when tested in combination with isoniazid (INH) in INH/rifampicin resistant strains and notably prevented the development of INH resistance. However, the intrinsic anti-*Mycobacterium* activity of epiroprim and combinations are too weak in order to develop the drug for this indication. Therefore, novel DHFR inhibitors would need to show much better activity than epiroprim against strains of *M. tuberculosis*. One example of a molecule with better activity than epiroprim against *M. tuberculosis* is the triazine DHFR inhibitor WR99210. WR99210 was shown to exhibit reasonable in vitro activity against *M. tuberculosis*, as well as against other mycobacteria and this structural class was further pursued, using a genetically modified *S. cerevisiae* strain for screening [34]. The selectivity of WR99210, however, is not sufficient for use as an antimicrobial agent with the major disadvantage being problems of poor gastrointestinal tolerance associated with the molecule. A series of analogs have been synthesized and tested and showed activity comparable to the lead molecule however to the best of our knowledge there is no further information pertaining to their development.

Importantly the three dimensional structure of the *M. tuberculosis* DHFR, complexed with several inhibitors, has been solved [6]. Although the general folding of this protein is similar to that of the human enzyme several important differences have been observed. Such differences may well be exploitable in the discovery of novel DHFR inhibitors. One example is the diaminopyrimidine K-130 (see Scheme 3), which when coupled to dapsone was found to potently inhibit the DHFR from *Mycobacterium lufu*, a model organism, and also had high in vitro and in vivo activity against *M. lufu*, and *M. leprae* [35]. The activities of K-130 are encouraging and time will tell as to whether further development of this compound will proceed.

In addition to WR99210 and K-130, a series of 2,4-diamino-5-deazapteridine derivatives that exhibit significant activity against recombinant *M. avium* DHFR have been reported



Scheme 3 – Structure of K-130 [35].

[36,37]. These compounds exhibit DHFR IC_{50}-values in the nM range and promising in vitro activities with MICs below 0.13 μg/mL. Furthermore, many of them were shown to be selective for the mycobacterial DHFR with selectivity ratios of >100 as compared with human DHFR. However, the activity of the compounds against *M. tuberculosis* is not as good as their activity against *M. avium* and although the overall characteristics of the compounds are encouraging further improvements in terms of anti-mycobacterial activity are required. *M. avium* as a target organism for new DHFR inhibitors was also pursued in a novel approach to use pharmacophores in a series of substituted pyrimidines [38]. It is clear that the search for novel molecules that exert their anti-mycobacterial activities through inhibition of DHFR represents an interesting development. Progress in the field has been good and represents potential promise in the future development of novel anti-mycobacterial drugs.

Due to various concerns, a vast amount of industry and governmental efforts have been introduced in the discovery of new agents for the treatment of anthrax, caused by *Bacillus anthracis*. This area has seen activities in the search for new drugable-like anthrax targets as well as in the area of second generation like molecules for treatment options that should go beyond the use of quinolones such as ciprofloxacin. Although trimethoprim is reported not to show activity against *B. anthracis*, investigators have recently described dihdyrophtalazine and biphenylpyrimidine DHFR inhibitors with DHFR IC_{50} values ranging from 46 to 404 nM. These compounds also exhibit MICs ranging from 12.5 to 128 μg/mL [39]. At the time of writing no further information concerning this interesting development is available.

7. Conclusions

The discovery and development of bacterial DHFR inhibitors in the last decade has continued to be an area of important efforts, both in academia and industry. The ubiquitous nature of this enzyme provided the basis to target several indication areas. This information coupled with the vast amount of knowledge pertaining from DHFR genomic sequences and crystal structures, the history of TMP and the rapid progress made with iclaprim will continue to attract both companies and academic institutions in the discovery of new and novel antibacterial compounds.

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