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Structure aided design of chimeric antibiotics

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ABSTRACT

The rise of antibiotic resistance is of great clinical concern. One approach to reducing the development of resistance is to co-administer two or more antibiotics with different modes of action. However, it can be difficult to control the distribution and pharmacokinetics of two drugs to ensure both concentrations remain within the range of therapeutic efficacy whilst avoiding adverse effects. Hybrid drugs, where two drugs are linked together with a flexible linker, have been explored, but the resultant large, flexible molecules can have poor bioavailability. We have developed a chimeric approach using click chemistry where the pharmacophores of two drugs are overlapped into a single smaller, more drug-like molecule. Design and selection of compounds were assisted by in silico structural docking. We prepared a series of compounds that include candidates showing activity against the targets of both trimethoprim; dihydrofolate reductase, and ciprofloxacin; DNA gyrase and topoisomerase IV. The resultant triazole containing molecules show modest, but broad spectrum activities against drug sensitive and resistant Gram-negative and Gram-positive bacteria, with no observable cytotoxicity.

Drug resistant microorganisms and the paucity of new antibiotics in development pose a serious and growing threat to human health.[1] Of particular concern are drug resistant Gram-negative bacteria that possess multiple mechanisms to acquire genetic material encoding antibiotic resistance. The recent outbreak of carbapenem resistant *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Escherichia coli* expressing the plasmid-encoded metallo-β-lactamase NDM-1 was a frightening example of this rapid global horizontal gene transfer.[2] One recent approach to combating resistant pathogens is the development of hybrid antibiotic molecules in which two drugs with different modes of action are joined together, often using a flexible linker, in an attempt to improve on traditional drug combination therapies. While this strategy combines two modes of action into one chemical entity, simplifying optimisation of the pharmacokinetics/pharmacodynamic (PK/PD) profile, efficacy at both targets is usually compromised. The hybrids often have a more flexible structure, thereby reducing target binding efficiency, and have a higher molecular weight, which negatively impacts both oral bioavailability and the ability to penetrate the outer membrane of Gram-negative bacteria.[3]

The development of linked hybrid antibiotic has been problematic to date, with only cadazolid (ACT-179811), an oxazolidinone and quinolone hybrid being actively developed in the clinic where it is being evaluated in Phase II trials for the treatment of *Clostridium difficile* infections.[4] Other recent hybrids where further development has been halted include MCB 3681, an oxazolidinone–quinolone hybrid that reached Phase I,[5] CBR-2092, a rifampicin–quinolone hybrid that completed Phase I, and TD-1792,[6] a vancomycin–cephalosporin hybrid that completed Phase II.[7]

We propose a progressive improvement of the hybrid antibiotic concept with a chimeric strategy.[5] Existing drugs are conceptually broken down into fragments and then recombined into a single, chimeric, drug-like molecule bearing the required pharmacophores for the different bacterial targets. This modification maintains the requisite molecular size and physicochemical properties that may be suitable for an efficacious, orally available Gram-negative antibiotic. Reassembly of the fragments into active molecules is aided by extensive molecular modelling, docking and structural studies.

To validate our approach, we have selected the benzyl pyrimidine (e.g., TMP and iclaprim) and the fluoroquinolone (FQ, e.g., ciprofloxacin (CIP)) classes of antibiotics as (i) both show broad spectrum activity against both Gram-negative and Gram-positive bacteria, (ii) drug synergy studies show that they have a additive and no antagonistic effect[8] (iii) X-ray crystal structures of benzyl pyrimidines, and FQs bound to their targets are available and, (iv) extensive SAR information is known for both classes.

Benzyl pyrimidines inhibit DHFR, an essential enzyme which is critical in the biosynthesis of purines and amino acids. DHFR catalyses the reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate using nicotinamide adenine dinucleotide phosphate oxidase (NADPH) as a co-factor. Numerous X-ray crystal structures of bacterial DHFR with various inhibitors have been published with the

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Protein DataBank (PDB) containing 133 entries to date, but lacking the DHFR of important pathogenic Gram-negative organisms. Homology models of DHFR from *K. pneumoniae*, *Pseudomonas aeruginosa* and *A. baumannii* were built using the crystal structure of *E. coli* DHFR (Pdb: 2ANO[13]) as template (Fig. 1; see Supplementary data for detail). Comparison of the DHFR active site residues from these bacteria revealed that they are very well conserved, showing the potential for broad spectrum activity against these pathogens.[14] Most importantly, bacterial DHFRs show only <30% homology with human DHFR versus 30–45% within bacterial species, providing a selectivity window for the benzyl pyrimidines.

Fluoroquinolones are known to predominantly target two similar and essential bacterial enzymes, DNA gyrase (topoisomerase II) and topoisomerase IV. DNA gyrase and topoisomerase IV both contain four subunits (2 GyrA and GyrB and 2 ParC and ParE) and are both classed as type II topoisomerases. DNA gyrase introduces negative supercoils into DNA, while topoisomerase IV catalyses ATP-dependent chromosome decatenation and relaxation.[9] The primary target of most quinolones in Gram-negative bacteria is DNA gyrase, while the primary target in Gram-positive bacteria is a combination of topoisomerase IV and DNA gyrase.[10] The exact nature of the quinolone interaction was first reported in 2009 by a X-ray crystal structures of *Streptococcus pneumoniae* ParC breakage-reunion and ParE TOPRIM domains stabilized by two FQs, moxifloxacin and clinafloxacin.[11] The cleaved DNA is thereby bound into the topoisomerase IV with the FQs intercalated into a gap of the DNA between the −1 and +1 nucleotide pairs. Due to this complex mode of inhibition a structural comparison between the mode of inhibition of Gram-positive and Gram-negative gyrases are not meaningful, hence no homology models have been attempted for the gyrases.

Published results[12] of a series of TMP-FQ hybrid style molecules synthesised using a linking strategy that did not interfere with the key pharmacophore activity sites of TMP and FQs showed promising activity against resistant strains (e.g., MP-1341, MP-1350, and MP-1362, Fig. 2). The activity profiles were equivalent, or better than equimolar mixtures of CIP with TMP.

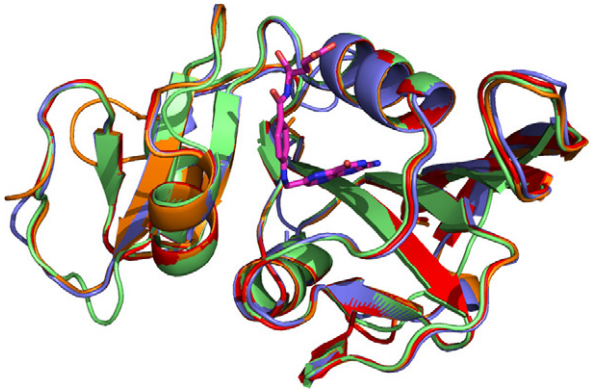
Translating these results into our chimeric strategy, we sought to design new compounds where the pharmacophores of both antibiotic compounds are combined into a single small molecule. The initial designs (Fig. 3) were done by evaluating the published SARs for TMP, iclaprim and FQs. In addition, the design varied the degree of overlap of the pharmacophores, exploring the effect of molecular weight, potentially improving their oral bioavailability and Gram-negative activity.[15]

The initial target compounds were synthesised via a napthyridine common intermediate, as either the ethyl ester **1** or free acid **2**, prepared by cyclisation of an enamino keto ester using the method of Bouzard[18] and Chu[19] or Shin[20] (Scheme 1). The resulting chloronaphthyridine was treated with an appropriate amine to form the desired chimeric antibacterial candidate.

Chimera **4** was prepared in three steps via methylamino piperazine **6** (Scheme 3).[21] Reduction of nitrile **5** to the desired amine **6** proved to be challenging due to instability of the intermediate imine, however, small quantities of the amine were obtained. With amine **6** in hand, the target molecule **4** was prepared using the published condensation method.[12] After preparative HPLC purification, only a small quantity of the desired product was obtained at moderate purity and the material was subjected to biological assay. Unfortunately, chimera **4** was found to be inactive against Gram-positive and Gram-negative bacteria (Table 1).

The lack of activity led us to believe that we had not included enough of the required pharmacophore of the DHFR inhibitors, and attention shifted to the less overlapped compound **3**. This compound was intended to represent the case where the two antibiotics are more representative of the respective pharmacophores. Precedent for the activity of aminobenzyl pyrimidines against DHFR was known.[22] Preparation of the TMP portion of the molecule was relatively straightforward (Scheme 2). The aldehyde group of 5-nitrovanillin **7** was protected as the dioxolane before catalytic transfer hydrogenation of the nitro group and cyclisation of the resulting amine onto the adjacent phenol was accomplished using dibromoethane.[23]

Deprotection of the dioxolane protected aldehyde **10** and cyclisation of the diaminopyrimidine using the standard methodology provided the desired amine **12**.[24] Surprisingly, attempts to form the diaminopyrimidine in the presence of the nitro group were unsuccessful. In contrast, condensation of amine **12** with napthyridine **2** proved to be much more problematic. Under standard conditions[12] only traces of compounds with the desired mass spectrum were observed by LC–MS. Attempts to isolate these materials via preparative HPLC were unsuccessful. The use of Buchwald-type



S. aureus	*E. coli*	*A. baumannii*	*P. aeruginosa*	*K. pneumoniae*
Leu_5	Ile_5	Val_{11}	Ile_8	Met_6
Ala_7	Ala_7	Ala_{13}	Ala_{10}	Ala_8
Leu_{20}	Met_{20}	Leu_{26}	Leu_{23}	Ile_{21}
Leu_{28}	Leu_{28}	Leu_{34}	Leu_{31}	Gln_{29}
Val_{31}	Phe_{31}	Phe_{37}	Phe_{34}	Phe_{32}
Lys_{32}	Lys_{32}	Lys_{38}	Lys_{35}	Lys_{33}
Thr_{46}	Thr_{46}	Thr_{52}	Thr_{49}	Thr_{47}
Ile_{50}	Ile_{50}	Met_{56}	Leu_{53}	Met_{51}
Leu_{54}	Leu_{54}	Leu_{60}	Leu_{57}	Leu_{54}
Phe_{92}	Ile_{94}	Ile_{107}	Ile_{104}	Ser_{97}
Gly_{94}	Gly_{96}	Gly_{109}	Gly_{106}	Gly_{99}
Phe_{98}	Tyr_{100}	Phe_{113}	Tyr_{110}	Tyr_{103}

Figure 1. Top: Homology models of DHFR enzymes from *E. coli* (red), *A. baumannii* (blue, 33% identity/52% similarity to *E. coli*), *P. aeruginosa* (orange, 44%/61%), *K. pneumoniae* (green, 32%/56%). The dihydrofolate substrate is shown in magenta bound at the centre of the enzymes. Bottom: Comparison on DHFR active site residues for pathogens of interest.

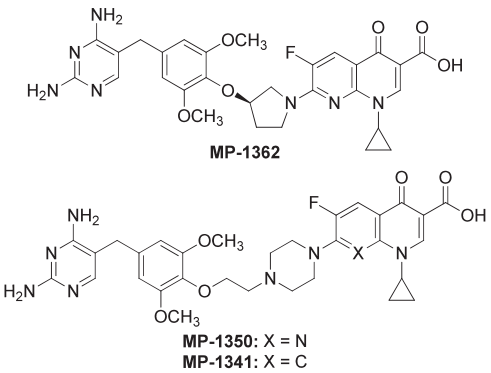


Figure 2. Published trimethoprim/fluoroquinolone hybrid structures.

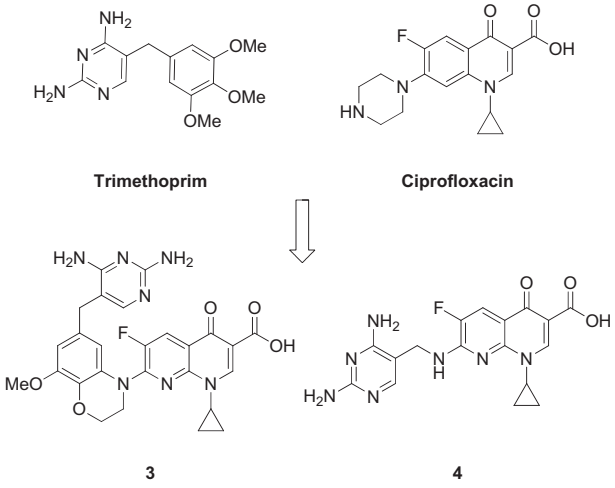
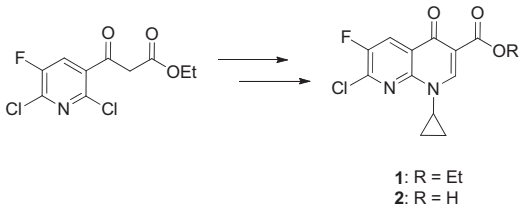


Figure 3. Initial target molecules, showing the template drugs.



Scheme 1. Common intermediate synthesis.

palladium chemistry[25] provided products in sufficient yield and quality for isolation by HPLC.

Small amounts of two distinct stable compounds were isolated (compounds **13** and **14**) showing the correct mass for the desired product; however, NMR analysis showed that neither was the desired material, and instead were the products of coupling at the pyrimidine NH_2 groups rather than the expected morpholino nitrogen. These compounds were assayed in our antibacterial panel (Table 1) but were found to be inactive. In terms of the antifolate activity, this result is not unexpected given that the diaminopyrimidine portion of the molecule is deeply buried in the enzyme and is the key pharmacophore of the drug class. Attempts to protect the diaminopyrimidine portion of **12** to allow reaction at the morpholino group were unsuccessful. To examine the probability of activity for this class of molecules, compound **12** was assayed and found to be only weakly active against two of the Gram-negative strains tested.

Examination of the difficulties encountered in the preparation of compounds **3** and **4** showed that the vigorous conditions required for coupling the amino pyrimidine and napthyridine components were causing decomposition of the products. To provide a more gentle method of combining the fragments, 'click' triazole preparation[26] was explored. At this stage various in silico methods were added to the design of new compounds, aimed to select compounds able to fit in to the binding sites of both DHFR and DNA gyrase, with the latter having a much more spatially restricted binding pocket.

Click chemistry is widely used in drug research for the synthesis of chemical libraries, and 1,2,3-trizoles containing drugs like tazobactam have reached the market. Compared to the previously published results[12] of TMP-FQ hybrid, the triazole compounds show slightly better drug-like properties, with a smaller molecular weight (500 instead of 650), improved predicted solubility ($-3.7 \log S$ compared to -5.2), fewer rotatable bonds (8 compared to 11), and reduced lipophilicity ($\log P$ of 0.9 compared to 2.9). Analysis of published crystal structures of proteins with triazole containing ligands shows mainly hydrophobic interactions with the protein, suggesting little electrostatic contribution of the triazole moiety to the overall binding energy.

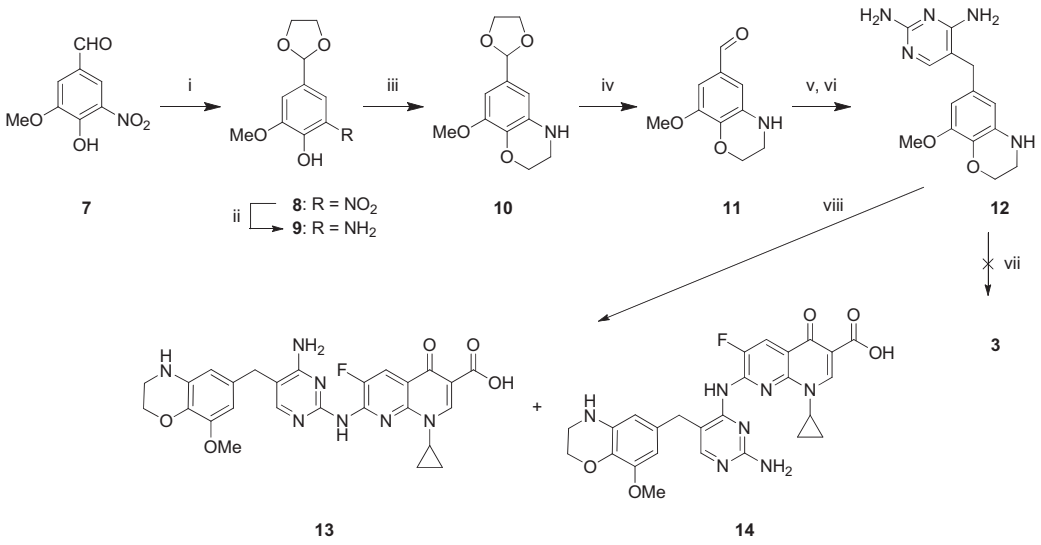
Virtual docking studies were undertaken using Gold v5.0.1,[17] using standard settings for docking and re-scoring with ChemScore. Binding sites were defined as residues within 1 nm of the corresponding inhibitor found in the crystal structure. While the docking into *E. coli* DHFR (Pdb: 2ANO[13]) binding site was straightforward, the docking into the *Staphylococcus aureus* topoisomerase II DNA-gyrase (Pdb: 2XCT[16]) required two additional settings in order to generate comparable docking poses in the FQ binding site: (i) scaffold constraint to the core of the ciprofloxacin inhibitor and (ii) definition of flexible side chains of Asp437, Arg458, Asn476 and Glu477. Compound poses not in the FQ binding site were not considered in the ranking. In both docking studies the inhibitors found in the crystal structures were used as an internal control, with both inhibitors ranking at the very top. The proposed compounds were ranked according to both virtual docking studies, eliminating compounds that did not fit, or that had low ranking in both docking studies (Fig. 4).

For the synthesis of those compounds, azide and alkyne analogues of the pyrimidine and napthyridine structures were required. The problematic imine stability observed during reduction of compound **5** (Scheme 1) was exploited[27] for preparation of the

Table 1
Assay results for chimeric compounds

Compound	*E. coli*[a] *s–s*[j]	G −ve (MIC μg/mL)			G +ve (MIC μg/mL)		Enzymatic assays (IC_{50} μM)		
		K. pneum[b]*s–r*	*P. aerug*[c]*s–r*	*E. coli*[d]*r–r*	*S. aureus*[e]*s–s*	*S. epiderm*[f]*s–r*	DNA Gyrase	TopoIV	DHFR
CIP[h]	⩽0.03	0.125	⩽0.03	>64	0.5	0.06	1.0	0.4	—[g]
TMP[i]	0.125–0.25	2	>64	>64	1	>64	—	—	0.1
TMP/CIP 1:1	⩽0.03	0.5	0.25–0.5	>64	0.5–1	0.125–0.25	—	—	—
4	>64	—	—	—	>64	—	—	—	—
12	8–16	32	>64	>64	8	>64	>250	>250	0.3
13	>64	>64	>64	>64	>64	>64	—	—	—
14	>64	>64	>64	>64	>64	>64	—	—	—
22	16	>64	>64	>64	32	16–32	166	>250	>250
24	>64	>64	>64	>64	>64	>64	>250	>250	>250
26	2	64	>64	>64	16	4	17	>250	—
28	0.5–1	16–32	>64	>64	4	4–8	48	4.5	>250
32	0.25	8	8–16	>64	0.25–0.5	0.5	10	4.4	>250
34	4	>64	64	>64	64	8	160	11	50

Notes: [a]ATCC 25922; [b]ATCC 700603; [c]ATCC 10145; [d]CIP and SXT resistant clinical isolate; [e]ATCC 25923; [f]NRS 60; [g]— = not tested; [h]ciprofloxacin; [i]trimethoprim; [j]sensitivity or resistance to CIP–SXT. SXT = co-trimoxazole (1:5 trimethoprim/sulfamethoxazole).



Scheme 2. Reagents and conditions: (i) ethylene glycol, pTSA, Dean–Stark; (ii) Pd/C, Et_3SiH; (iii) K_2CO_3, dibromoethane, DMF; (iv) H_3O^+; (v) 3-morpholinopropanenitrile, NaOMe; (vi) guanidine·HCl, NaOMe; (vii) 2, Et_3N, TMSCl; (viii) 1, $Pd_2(dba)_3$, DavePhos, Cs_2CO_3 then LiOH

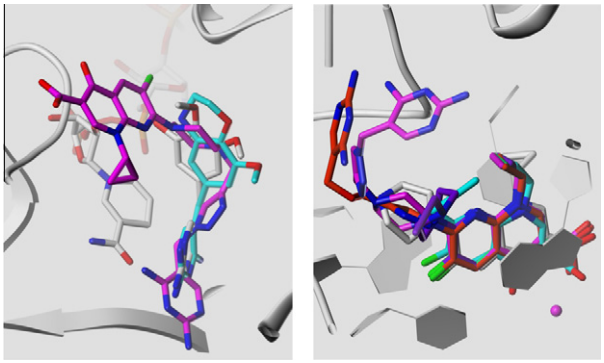
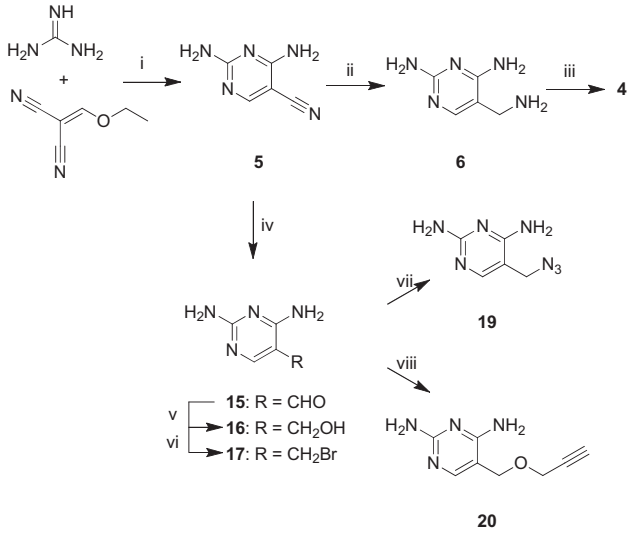


Figure 4. Left: Docked pose for TMP (grey), **12** (cyan) and **34** (magenta) into DHFR. Right: Docked pose for CIP (grey), **26** (cyan), **28** (red), **32** (pink) and **34** (magenta) into DNA gyrase.



Scheme 3. Reagents and conditions: (i) NaOEt, rt, 53%; (ii) H_2/Raney nickel;[21] (iii) 2, Et_3N, TMSCl: (iv) Raney nickel, HCOOH, reflux, 3 h; (v) $NaBH_4$, MeOH, rt, 5 h; (vi) HBr/HOAc 60 °C, 2 h, giving HBr salt; (v) NaN_3, DMF, 80 °C, 3 h; (vi) propargyl alcohol.

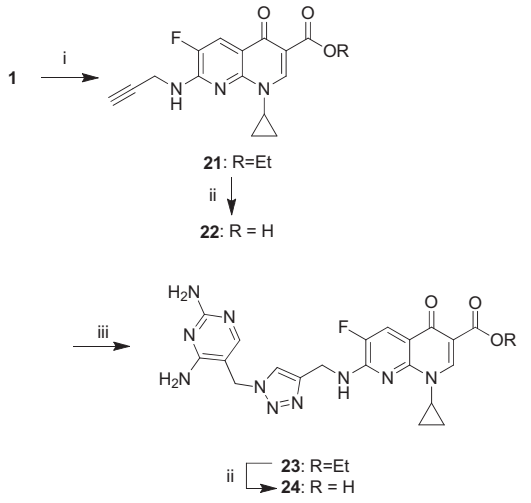
pyrimidine analogues where nitrile **5** was reduced to the imine and hydrolysed to aldehyde **15**. The aldehyde was then reduced with sodium borohydride to alcohol **16** which was converted to the unstable bromide **17** as the hydrobromide salt. This material was readily converted to the azide **19** or propargyl ether **20** using sodium azide or propargyl alcohol, respectively (Scheme 3). The napthyridine analogues were readily prepared from compound **1** by heating with propargyl amine or sodium azide to give alkyne **21** and azide **25**, respectively. A click reaction[28] produced the desired triazoles **24** and **28** after hydrolysis of the ethyl esters (Schemes 4 and 5).

To extend the pharmacophore of the fluoroquinolone portion of the molecule, an additional compound bearing a piperazine ring between the napthyridine and pyrimidine moieties was desired. Beginning with mono-Boc protected piperazine, alkyne **31** was prepared and reacted with azide **19** to produce chimera **34** after ester hydrolysis (Scheme 6).

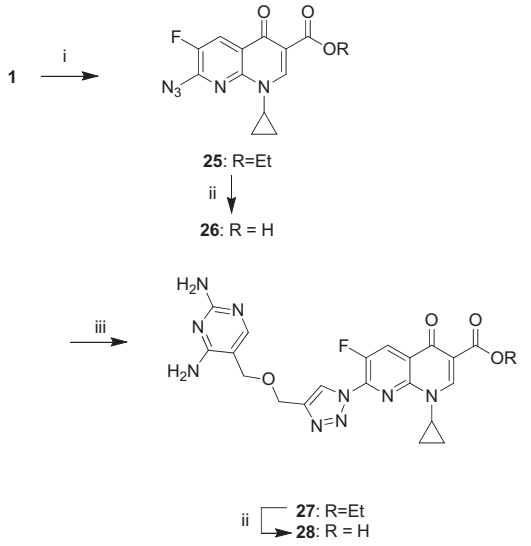
Biological assessment of the compounds was performed in three phases using standard assay procedures, (i) antibacterial MIC assays against Gram-positive and Gram-negative organisms, using sensitive and resistant strains, (ii) enzymatic assays against topoisomerase IV, DNA gyrase and DHFR[9b,c] and (iii) cytotoxicity assay against HepG2 and HEK293 cell lines (see Supplementary data for assay detail).

Of the compounds tested, only **21** showed cytotoxicity up to the maximum tested concentration of 100 μM, against either HepG2 or HEK293 cells. Compound **21** showed a CC_{50} of around 80 μM for both cell lines. In general, the ethyl esters (**21**, **23**, **25**, **27**, **31**, and **33**) showed no activity in either the cell based MIC assays or in the enzymatic inhibition assays. Only compound **23** showed a weak (80%) inhibition of DHFR at the highest concentration tested (250 μM), but no effect on the growth of the bacteria. On the other hand, FQ like intermediates (**22**, **26** and **32**) showed, as to be expected, some activity in the DNA gyrase and topoisomerase IV assays, and some activity in the cell based assays, but always less than is observed for CIP. The piperazine containing intermediate (**32**), which is the structurally closest to ciprofloxacin, showed the best activity profile in enzyme and cell based assays. Similarly, the TMP-like intermediate **12** showed activity against DHFR that was translated into mild in vitro activity against sensitive Gram-positive and Gram-negative strains. Interestingly, **12** showed activity against the SXT resistant *K. pneumoniae* strain.

From the designed compounds only **28** and **34** showed some enzymatic and in vitro activity, whereas **24** did not show any



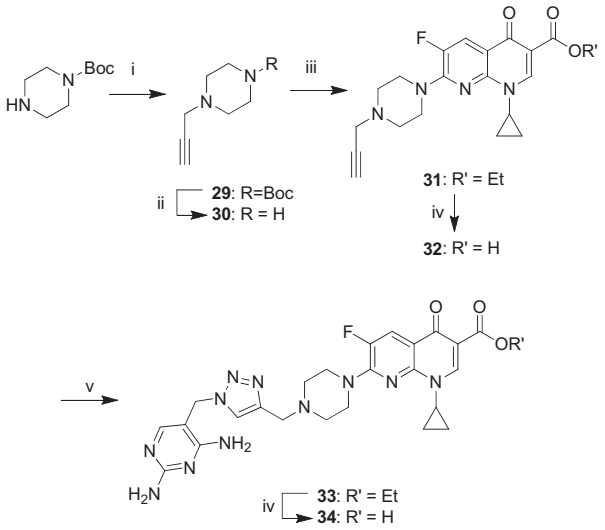
Scheme 4. Reagents and conditions: (i) Propargyl amine, THF, Et_3N, reflux 12 h; (ii) aq 2 N NaOH, MeOH, rt, 3 h; (iii) **19**, $CuSO_4$, sodium ascorbate, $BmPy_2$, rt, 12 h.



Scheme 5. Reagents and conditions: (i) NaN_3, THF, reflux, 5 h; (ii) aq 2 N NaOH, MeOH, rt, 3 h; (iii) **20**, $CuSO_4$, sodium ascorbate, $BmPy_2$, rt, 12 h.

activity at all. Only compound **34** satisfied the design criteria of showing inhibitory effect against DHFR, DNA gyrase and topoisomerase IV. Unfortunately this compound's ability to inhibit the growth of bacteria was not as effective as the mixture of ciprofloxacin and trimethoprim, with modest to poor activity against *E. coli*, SXT resistant *P. aeruginosa* and a glycopeptide resistant strain of *Staphylococcus epidermidis*. On the other hand **28** exhibited better ability to inhibit CIP sensitive bacteria, including sensitive *E. coli*, SXT resistant *K. pneumoniae*, sensitive *S. aureus* and glycopeptide resistant *S. epidermidis*. This selectivity profile for compound **28** matches the observed enzymatic assays, as **28** shows no inhibitory effect on DHFR, but modest inhibition of DNA gyrase and topoisomerase IV.

The in silico docking experiments gave some insights, but the scoring of individual compounds did not reflect the inhibitory activity against DHFR well, even though the reference compound TMP had the best score and overlayed with its crystal structure. Nevertheless, the poses of the compounds in the binding site were able to give some indications on possible reasons for their activity. Compound **34** appears to make additional hydrophobic interactions by placing the quinolone group into a hydrophobic groove,



Scheme 6. Reagents and conditions: (i) Propargyl bromide; (ii) TFA; (iii) **1**, THF, Et_3N, reflux, 12 h; (iv) aq 2 N NaOH; (v) **19**, $CuSO_4$, sodium ascorbate, $BmPy_2$, rt, 12 h

close to Trp_{22} and Lys_{28}, which the shorter **24** and **28** are not able to reach.

Docking experiments using the *S. aureus* topoisomerase II DNA-gyrase better reflected the experimental results with the most active compounds (**28**, **32** and **34**) among the top scoring compounds, while the non-active compounds (**23** and **24**) ranked lowest. Docking poses of the compound in the binding site indicated that active compounds have the pyrimidine located in a pocket around Arg_{458} and Asp_{437}. Compound **24** exhibited a conformation with the pyrimidine close to DNA with no protein interaction possible.

The work presented here demonstrates the incorporation of the pharmacophores from two active compounds with variant modes of action into one molecule. Limitations in the synthetic tractability of the strategy necessitated a shift from a pure chimeric design towards a more hybrid approach by increasing the linker spacer between the two pharmacophores. The in vitro activity relationships observed perhaps reflect the structural constraints in binding sites of the targets and the relative large size of the individual original starting pharmacophores, rendering the linker a crucial part of the molecule implicated in activity. The compounds reported here show modest in vitro activity, but they do posses more drug-like chemical properties and show no cytotoxicity. The work further illustrates the use of click chemistry and provides an example of triazole containing compounds with antibacterial activity, which opens the possibilities of using click chemistry more widely in the discovery of new antibiotics. Compounds **28** and **34** show potential as starting points for further optimisation studies, especially against antibiotic-resistant strains.

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Supplementary data

Supplementary data (key experimental details for the synthesis of new compounds and biological analyses) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2012.02.019.

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