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HTS, Chemical Hybridization, and Drug Design Identify a Chemically Unique Antituberculosis Agent–Coupling Serendipity and Rational Approaches to Drug Discovery

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Tuberculosis (TB) is presently regarded as one of the most dangerous infectious diseases worldwide and one of the major AIDS-associated infections. The simultaneous presence of HIV infection, the spread of drug-resistant strains of *Mycobacterium tuberculosis*, and the lack of compliance with the lengthy complex therapies often complicate its treatment.[1] Overall, one-third of the world's population is currently infected with the TB bacillus and it has been estimated that 1.7 million died in 2004.[2] The current TB chemotherapy regimen requires patients to take three to four drugs (isoniazid, rifampin, pyrazinamide, and ethambutol) for a minimum of six months. Of considerable concern is the fact that no new TB drugs have been discovered in the past 40 years.[3] The search for new antituberculosis agents is critical to global healthcare. Of particular need is the identification of improved drugs that can shorten the present six-month therapy regimen, and that can also be combined with antiretroviral drugs for HIV patients.

To identify new chemical scaffolds that could be used as a starting point in the creation of new TB drugs, we screened a 50 000 compound library (NOVACore Chembridge) against *Mycobacterium tuberculosis* rather than singling out a specific target. This whole cell screening would allow for the identification of compounds able to penetrate the cell wall of the bacterium while also allowing for the identification of compounds working through more than a single molecular target. The complete results from this HTS program will be published in due course. One scaffold from this library is represented by the structure shown in Figure 1, in which an aromatic ring fused to a second aromatic or nonaromatic ring comprises the R^1 group of this substituted isoxazole-3-carboxamide. This series was comprised of 2103 isoxazoles in which the carboxamide group is derived from either a secondary or tertiary amine (Scheme 1).

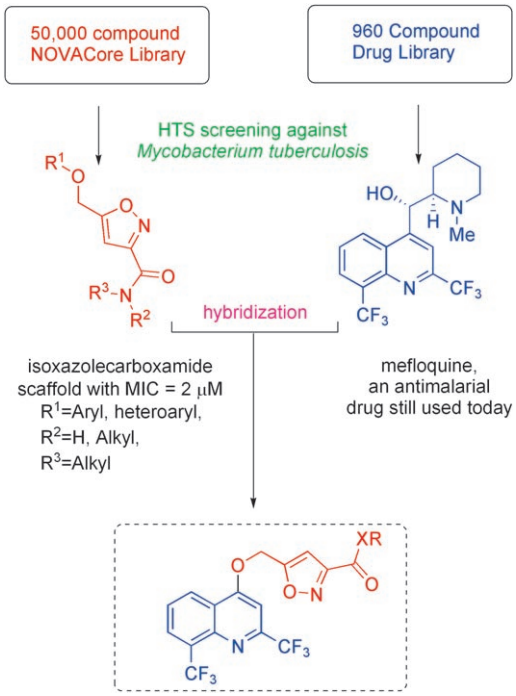

Figure 1. Summary of the HTS campaigns and subsequent hydridization strategy.

Moreover, previously we had carried out another HTS campaign in which we had investigated the anti-TB activity of a known drug library of 960 compounds. In that work the well known antimalarial drug mefloquine emerged as a possible lead candidate, and a number of analogues were prepared and tested based upon this starting compound.[4] Accordingly, we replaced the piperidine ring of mefloquine with piperazine and pyridine rings and with substituted aromatic rings. As some of these analogues showed better anti-TB activity than mefloquine itself, our prior work demonstrated that some structural modifications could be tolerated within this region of the molecule. This prior SAR information together with the screening data from the NOVACore library now suggested that it would be logical to combine certain chemical features of these two chemically diverse series to arrive at hybrids of possibly even better activity.

In particular, as one of the present investigators had previously conducted extensive research in the area of nitrile oxide cycloaddition chemistry[5] (NOC chemistry, which is mechanistically related to azide cycloaddtion strategies commonly referred to as click chemistry) and had previously reported on important methodology using the reagent CEFNO[6] (a simple precursor of which is available from Aldrich), it became interesting to attach the quinoline moiety of mefloquine to the isoxazole scaffold as the R^1 substituent. To test this idea, we first prepared 4-prop-2-ynyloxy-2, 8-bis-trifluoromethylquinoline (**2**)

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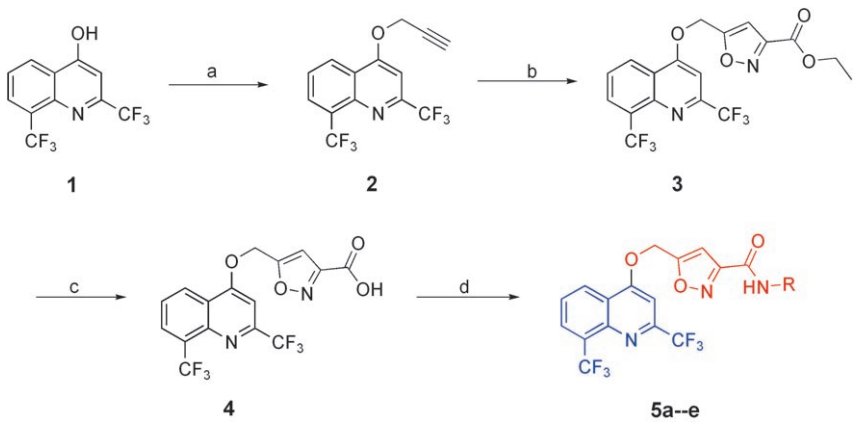
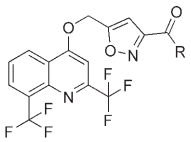


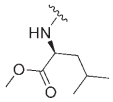
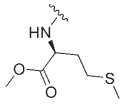
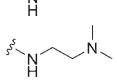
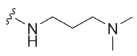
Scheme 1. Reagents and conditions: a) propargyl bromide, K_2CO_3, acetone, reflux, overnight, 95%; b) ethyl chlorooximidaocetate, Et_2O, TEA, 6 h through syringe pump, 54%; c) LiOH, THF:H_2O:MeOH = 3:1:1, RT, 4 h, 90%; d) RNH_2,EDC, HOBt, DIEA, DCM, RT, overnight.

from 2, 8-bis-trifluoromethylquinolin-4-ol (**1**) by reaction with propargyl bromide in the presence of potassium carbonate in anhydrous acetone. Next, the NOC reaction was carried out by exposing the dipolarophile **2** to ethyl chlorooximidoacetate in the presence of base to afford 5-(2, 8-bis-trifluoromethylquinolin-4-yloxymethyl) isoxazole-3-carboxylic acid ethyl ester (**3**). This ester was hydrolyzed with lithium hydroxide to yield the corresponding acid **4**, which was then converted to several different amides **5a**–**e** by use of EDC. The hybrid analogues created using this strategy are shown in Table 1.

All the compounds were evaluated for their activity against the *M. tuberculosis* strain $H_{37}Rv$ by the microplate Alamar Blue assay[7] (MABA) and low oxygen recovery assay[8] (LORA). The in vitro cytotoxicity assay was performed as previously described.[9] Surprisingly, compound **3** (Table 1) was found to be the best compound in this series, as it has excellent antituberculosis activity (MABA MIC = 0.9 μM), and it is thus only ninefold less potent than the TB drug rifampin, and about 15-fold more potent than mefloquine. In the LORA assay, the MIC (12.2 μM) is similar to that of mefloqine. Moreover, compound **3** shows no cytotoxicity toward either Vero cells[10,11] or J744.1[11] cells (data not shown), suggesting the likelihood that the compound would not show adverse toxicity. As compound **3** has an excellent selectivity index (SI), these results encouraged us to investigate other analogues of this compound. Thus, five amides of this ester were prepared whose selection was based upon the previous SAR knowledge of mefloquine and the HTS library. Amide **5a** prepared from L-leucine methyl ester, exhibits a modest MIC in the MABA assay (7.5 μM)

Table 1. In vitro activity against *M. tuberculosis* $H_{37}Rv$.



Comppd.	Structure R =	MIC [μM] MABA	MIC [μM] LORA	IC_{50} [μM] Vero cells	SI (Selectivity Index) MABA	SI (Selectivity Index) LORA	CLog *P*[a]
1	Mefloquine	13	7	11	0.9	1.6	3.6
RMP	Rifampin	0.1	2	122	1220	61	–
3		0.9	12.2	> 128	> 75.3	> 10.5	4.41
4		> 128	> 128	> 128	–	–	3.96[b]
5a		7.5	94.3	> 128	> 17.07	> 1.3	5.29
5b		> 128	> 128	> 128	–	–	4.47
5c		> 128	> 128	> 128	–	–	4.42
5d		7.9	15.9	> 128	> 16.2	> 8.1	3.63
5e		52.5	112.3	12.3	–	–	4.12

[a] KOWWIN from http://www.vcclab.org/lab/alogps/start.html. [b] $pK_a = 3.75$ from http://ibmlc2.chem.uga.edu/sparc/, CLog *D* = Log *P* − Log (1 + 10*(pH-pK_a)) = 3.96 − log (1 + 10*(7.4 − 3.75)) = 2.386.

whereas the related analogue **5b** prepared from L-methionine methyl ester does not show any activity. We also synthesized the dimethylaminoalkylamides **5d** and **5e** with linkers of different length. The compound **5d** with a two-carbon linker has a MABA MIC that is better than that of **5e**, but these compounds are still less potent than the ester **3**.

Whereas it will be valuable to further explore the SAR around **3**, its MIC is still well within the range that may qualify it as a possible lead candidate. To profile this compound further we tested its MIC in the presence of 5 mg mL^{-1} fetal bovine serum (FBS) in the MABA assay to assess whether plasma protein binding would lead to a diminution in its activity. Its MIC in the presence of FBS does not differ significantly from the MIC determined in the absence of FBS (1.54 μM versus 0.9 μM).

As mentioned above, the ideal antituberculosis agent should have the capacity to be used in combination with antiretroviral drugs for HIV patients. As many HIV antivirals such as indinavir, nelfinavir, ritonavir, and saquinavir are substrates for CYP3A4, it would be best that compound **3** act neither as an inducer nor as an inhibitor of CYP3A4. We therefore used the Invitrogen Vivid CYP3A4 Green Screening kit to test for the effect of **3** on this oxidative enzyme. Compound **3** shows no inhibition of CYP3A4 in this assay.

We also note that the corresponding acid form **4** of our most active compound **3** fails to show any antituberculosis activity. Whereas it is likely that the charged acid **4** is unable to penetrate the wax-like cell wall of the TB bacterium, we can not rule out the possibility that the ester serves as a prodrug, and that it is converted into the active acid form once inside the bacterium. As such, it will be valuable to investigate this possibility further, as we may be able to obtain even more active drug candidates following this idea.

In summary, the present work reveals a valuable strategy to pursue in the quest to identify new chemicals for TB therapy. In this work, the screening of two chemical libraries, one purely synthetic, and the other representing a drug library, led to the design of a hybrid structure demonstrating biological properties conducive to its advancement to higher level animal studies. In this regard, we note that once daily oral dosing at 200 mg kg^{-1} for 5 days resulted in no observable toxicity or weight loss. Evaluation of this hybrid molecule in infected animals as well as efforts to identify its biological target(s) are currently in progress. The present findings hold considerable promise in identifying new medicines to curb the unnecessary loss of life globally, and further demonstrate the power of NOC chemistry.

Keywords: isoxazole · mefloquine · MIC · nitrile oxide cycloaddition · tuberculosis

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