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Bioorganic & Medicinal Chemistry 13 (2005) 6663–6671
Bioorganic & Medicinal Chemistry

Synthesis and in vitro anti-mycobacterial activity of 5-substituted pyrimidine nucleosides

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Received 14 January 2005; revised 16 July 2005; accepted 18 July 2005
Available online 1 September 2005

Abstract—*Mycobacterium tuberculosis* and *Mycobacterium avium* infections cause the two most important mycobacterioses, leading to increased mortality in patients with AIDS. Various 5-substituted 2′-deoxyuridines, uridines, 2′-*O*-methyluridine, 2′-ribofluoro-2′-deoxyuridines, 3′-substituted-2′,3′-dideoxy uridines, 2′,3′-dideoxyuridines, and 2′,3′-didehydro-2′,3′-dideoxyuridines were synthesized and evaluated for their in vitro inhibitory activity against *M. bovis* and *M. avium*. 5-(C-1 Substituted)-2′-deoxyuridine derivatives emerged as potent inhibitors of *M. avium* (MIC_{90} = 1–5 μg/mL range). The nature of C-5 substituents in the 2′-deoxyuridine series appeared to be a determinant of anti-mycobacterial activity. This new class of inhibitors could serve as useful compounds for the design and study of new anti-tuberculosis agents.

1. Introduction

Thirty-three percent of the world's population is infected by *Mycobacterium tuberculosis*. Every year eight million new cases appear and 2–3 million deaths occur due to tuberculosis (TB) around the world.[1–3] In the beginning of the 1900s, TB was the most common cause of death due to an infectious disease. There was a gradual decline in the number of cases from the early 1950s. However, there has been a resurgence in the incidence of TB since 1984. In 1993, the World Health Organization (WHO) declared TB as a medical emergency. A number of factors have contributed to the resurgence of TB, such as synergy between TB and the acquired immune deficiency syndrome (AIDS) epidemic, non-compliance due to severe side effects of the available therapy, development of multi-drug resistant clinical isolates, neglected TB control in many countries, and socio-economic trends.[1,4,5] Two groups of mycobacteria *M. tuberculosis* and *Mycobacterium avium* pose a significant threat of TB in human immunodeficiency virus (HIV) infected patients and are often responsible for their death.[6] Besides HIV infection, other individuals with compromised immune systems are also highly susceptible to TB infection and reactivation causing disease. Reactivation of TB can be caused by immunodeficiency, use of immunosuppressive drugs, aging, alcohol, and drug abuse.

Bacillus Calmette Guerin (BCG) is used as a vaccine; however, it does not reduce the transmission of TB and is not effective for TB infected patients.[7] BCG is an attenuated strain of *Mycobacterium bovis* (*M. bovis*), which is more than 98% homologous to *M. tuberculosis* and therefore is closely related to *M. tuberculosis*. *M. bovis* infections in HIV patients are also increasingly being realized as causing TB.

Mycobacterium avium complex (MAC) infections, in particular *M. avium* infections, are one of the most serious complications among patients with AIDS in many developing as well as developed countries such as the United States and European countries.[8,9] *M. avium* infections are disseminated rather than restricted to the lungs. Clinical management of MAC infections is very difficult, because many of the first-line anti-TB drugs are ineffective against it.[8,9] New macrolides, such as clarithromycin and azithromycin, are used for the treatment of MAC; however, resistance occurs at such

Keywords: Tuberculosis; Heterocycle; Anti-microbial agent.
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doi:10.1016/j.bmc.2005.07.046

a rate with macrolide therapy that single drugs are inadequate.[10,11] Therefore, development of new antimycobacterial agents with potent activity against *M. avium* is urgently required.

The control and management of mycobacterial infections are highly important not only for the survival of AIDS patients, but also for the people who are or will be infected with multi-drug resistant bacteria, harbor strains that develop resistance after previous drug treatment, immunocompetent or immunocompromised people in proximity of HIV patients, and patients with MAC infection. There is an ever-increasing threat of drug-resistant TB appearing as an epidemic in many countries, particularly because no new classes of TB-specific drugs have been developed since the rifampicin in 1967.[3]

The investigation of new classes of anti-TB agents is a high priority. A new class of agents which work by different mechanisms than current drugs, and are not cross-resistant with them, is likely the best long-term prospect to augment current therapy, address the resistance crisis, and meet the global health emergency.

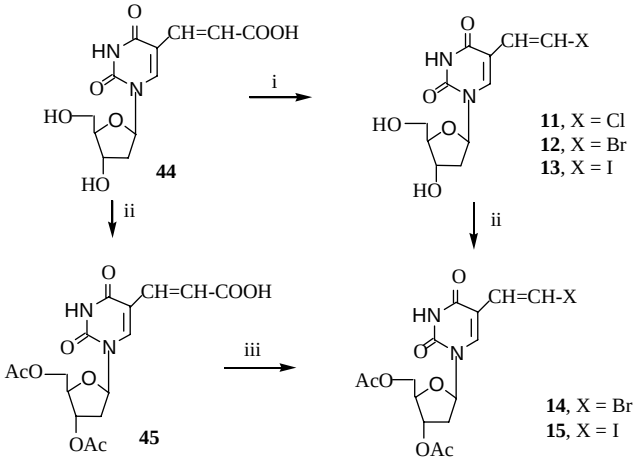
In the search for new anti-tuberculosis agents, our recent studies have focused on the design, synthesis, and development of unnatural pyrimidine nucleosides as potential new chemotherapeutic agents for mycobacterial infections. In the present investigation, we have synthesized and evaluated several classes of unnatural deoxyribose, ribose, and dideoxyribose pyrimidine nucleoside derivatives to determine the effect of size and electronegativity of groups at C-5, C-2′, and C-3′ on the antimycobacterial activity against *M. bovis* and *M. avium*. It was postulated that unnatural pyrimidine nucleosides can specifically target the mycobacterial enzymes, such as *Mtb* DNA polymerase, involved in their nucleic acid synthesis by acting as their substrates and/or inhibitors, and inhibit the mycobacterial DNA and/or RNA synthesis. Of the newly investigated compounds, 5-(1-hydroxyethyl)-2′-deoxyuridine (**41**), 5-(1-fluoro-2-chloroethyl)-2′-deoxyuridine (**42**), and 5-(1-fluoro-2-bromoethyl)-2′-deoxyuridine (**43**) were found to be selective inhibitors of *M. avium* multiplication. 5-(1-Substituted alkyl) pyrimidine nucleosides are described here for the first time as potential antimycobacterial agents against *M. avium*.

2. Chemistry

The target 5-halo derivatives of 2′-deoxyuridine (**2**–**4**) and uridine (**17**,**18**) were synthesized by the reactions of 2′-deoxyuridine and uridine, respectively, with *N*-bromo (or chloro) succinimide or iodine monochloride and sodium azide at 25–45 °C using the efficient halogenation procedures reported by us earlier.[12] 5-Vinyl-2′-deoxyuridine (**10**) was readily prepared by the palladium acetate–triphenyl phosphene-catalyzed reaction of 5-iodo-2′-deoxyuridine with vinyl acetate according to the method of Rahim et al.[13] The reaction of (*E*)-5-(2-carboxyvinyl)-2′-deoxyuridine (**44**)[14] with *N*-chlorosuccinimide in dioxane/water (3:7, v/v) provided (*E*)-5-(2-chlorovinyl)-2′-deoxyuridine (**11**) in 45% yield. In comparison, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (**12**) was obtained in quantitative yield (98%) by the reaction of **44** with *N*-bromosuccinimide in THF and water, as shown in Scheme 1. This procedure resulted in an improved synthetic route for **12** over the previously reported method.[15] It was observed that the use of THF resulted in the increased yield. (*E*)-5-(2-Iodovinyl)-2′-deoxyuridine (**13**) and (*E*)-5-(2-bromovinyl) uridine (**21**) were prepared starting from (*E*)-5-(2-carboethoxyvinyl)-2′-deoxyuridine and (*E*)-5-(2-carboethoxyvinyl) uridine, respectively.[16]

Nucleoside **12** was converted to the corresponding 3′,5′-di-*O*-acetylated derivative (**14**) by the reaction of acetic anhydride in pyridine, in 99% yield (Scheme 1). However, difficulties were encountered in obtaining good amounts of 3′,5′-di-*O*-acetyl-(*E*)-5-(2-iodovinyl)-2′-deoxyuridine (**15**) by direct acetylation of **13**. Therefore, an alternative procedure was used for the synthesis of target compound **15**. The reaction of (*E*)-5-(2-carboxyvinyl)-2′-deoxyuridine (**44**) with acetic anhydride in pyridine yielded the corresponding acylated derivative (**45**), which was subsequently reacted with *N*-iodosuccinimide and potassium acetate in dry *N*,*N*-dimethylformamide at room temperature to provide the desired 3′,5′-di-*O*-acetyl-(*E*)-5-(2-iodovinyl)-2′-deoxyuridine (**15**) in 55% yield (Scheme 1).

1-(2′-Fluoro-2′-deoxy-β-D-ribofuranosyl) uracil (**23**) and 1-(2′-Fluoro-2′-deoxy-β-D-ribofuranosyl) thymine (**26**) were prepared, according to the published procedure.[17] The 2′-5-difluoro analog (**24**) was obtained by the electrophilic reaction of **23** with nitrogen-diluted fluorine in acetic acid.[18] The reaction of **23** with iodine monochloride in methanol iodinated the uracil ring at C-5, providing 5-iodo-2′-ribofluoro-2′-deoxyuridine (**25**) in 60% yield.[19] (*E*)-5-(2-Iodovinyl)-2′-ribofluoro-2′-deoxyuridine (**27**)[20] was synthesized from (*E*)-5-(2-carboxyvinyl)-2′-ribofluoro-2′-deoxyuridine by treatment with *N*-iodosuccini-



Scheme 1. Reagents and conditions: (i) *N*-chlorosuccinimide, dioxane/H_2O (3:7), glacial acetic acid 25 °C (**11**); *N*-bromosuccinimide, THF/H_2O, 25 °C (**12**); (ii) acetic anhydride, pyridine, 25 °C; (iii) *N*-iodosuccinimide, dry DMF, potassium acetate 25 °C.

mide in *N*,*N*-dimethylformamide; that itself was obtained from (*E*)-5-(2-carboethoxyvinyl)-2′-ribofluoro-2′-deoxyuridine by alkaline hydrolysis.[20] The latter was prepared by the reaction of **25** with ethyl acrylate in acetonitrile in the presence of palladium (II) acetate, triphenyl phosphine, and triethylamine.[19]

3′-Azido-2′,3′-dideoxyuridine (**28**) and 3′-fluoro-2′,3′-dideoxyuridine (**30**) were synthesized using the method of Lin and Mancini[21] Previously published methods of Herdewijn and co-workers[22] and Balzarini et al.[23] were used to synthesize 3′-azido-2′,3′-dideoxythymidine (**29**), 3′-fluoro-2′,3′-dideoxythymidine (**31**), 2′,3′-didehydro-2′,3′-dideoxythymidine (**39**), 5-chloro-3′-fluoro-2′,3′-dideoxyuridine (**32**), 5-bromo-3′-fluoro-2′,3′-dideoxyuridine (**33**), and 5-fluoro-3′-fluoro-2′,3′-dideoxyuridine (**34**).

A variety of methods have been developed for the preparation of 2′,3′-didehydro-2′,3′-dideoxy nucleoside analogs. Van Aerschot et al.[24] reported the synthesis of 5-chloro-2′,3′-didehydro-2′,3′-dideoxyuridine (**46**) by chlorination at the C-5 position of 5′-acylated-2′,3′-didehydro-2′,3′-dideoxyuridine with *N*-chlorosuccinimide in pyridine at 100 °C. In contrast, iodination of 2′,3′-didehydro-2′,3′-dideoxyuridine (**47**) using iodine in the presence of the oxidizing agent, iodic acid, afforded low yields of desired 5-iodo-2′,3′-didehydro-2′,3′-dideoxyuridine (**40**). Efficient iodination of pyrimidine nucleosides at the C-5 position of the uracil ring has been described by Robins and Barr[25] using iodine monochloride in organic solvents. However, similar reactions employing 2′,3′-didehydro-2′,3′-dideoxyuridine afforded **40** as a minor compound with 5-iodouracil as the major product.

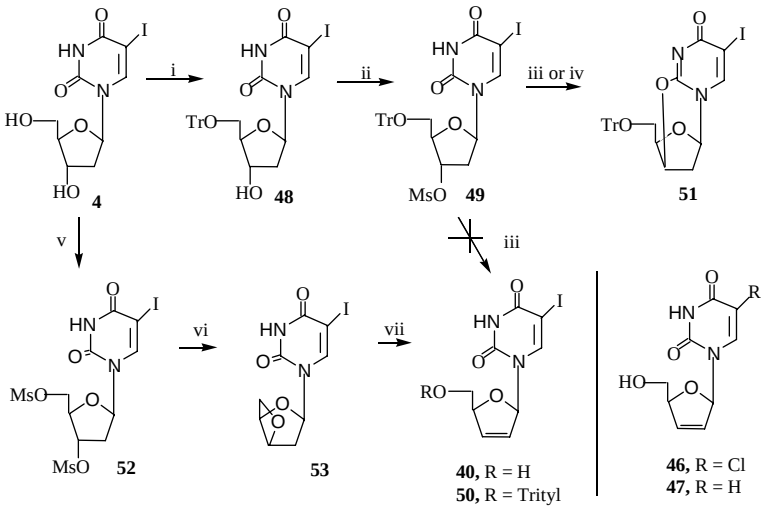
2′,3′-Didehydro-2′,3′-dideoxythymidine (**39**) was readily prepared by the reaction of 3′-*O*-mesyl-5′-*O*-tritylthymidine with tetrabutylammonium fluoride (TBAF) in THF at room temperature.[22] In contrast, employing similar reaction conditions with 3′-*O*-mesyl-5′-*O*-trityl-5-iodouridine (**49**) did not provide the desired 2′,3′-dihydro-2′,3′-dideoxy-5′-*O*-trityl-5-iodouridine (**50**), whereas 2,3′-anhydro-5-iodo-5′-*O*-trityl-2′-deoxyuridine (**51**) was obtained in 67% yield. The structure of compound **51** was confirmed further by comparing the ^{1}H NMR spectrum of the authentic sample, prepared by the reaction of **49** with sodium hydroxide in ethanol (method B).

We now report the synthesis of 2′,3′-didehydro-2′,3′-dideoxy-5-iodouridine (**40**) in excellent yield by a convenient route using 3′,5′-di-*O*-mesyl-5-iodo-2′-deoxyuridine (**52**) that was obtained in 83% yield by the reaction of 5-iodo-2′-deoxyuridine (**4**) and methanesulfonylchloride in pyridine at 0 °C. The reaction of **52** with sodium hydroxide in H_2O at reflux yielded 3′,5′-anhydro-5-iodo-2′-deoxyuridine (**53**) in 30% yield, which on treatment with potassium *tert*-butoxide in dry DMSO at room temperature provided 2′,3′-didehydro-2′,3′-dideoxy-5-iodouridine (**40**) in 75% yield (Scheme 2).

5-(1-Hydroxyethyl)-2′-deoxyuridine (**41**) was obtained by the treatment of **10** with aqueous hydrochloric acid.[26] The 5-(1-fluoro-2-chloroethyl)-(**42**) and 5-(1-fluoro-2-bromoethyl)-3′,5′-di-*O*-acetyl-2′-deoxyuridine (**43**) were prepared from the respective 5-(1-hydroxy-2-chloroethyl)- and 5-(1-hydroxy-2-bromoethyl)-3′,5′-di-*O*-acetyl-2′-deoxyuridines upon treatment with DAST in dichloromethane.[27] The latter were synthesized by the regiospecific reaction of 5-vinyl-3′,5′-di-*O*-acetyl-2′-deoxyuridine with HOX (X = Cl, Br).[27]

3. Results and discussion

All the test compounds were evaluated in vitro against *M. bovis* and *M. avium* by the microplate alamar blue assay (MABA)[28] at 1–100 μg/mL concentrations. Rifampicin and clarithromycin were used as reference standards. The results are given in Table 1. The 5-substituted pyrimidine nucleoside derivatives, modified in the sugar and/or base moiety, evaluated here for their antimycobacterial effect can mainly be divided into four different structural classes: (i) 2′-deoxyribose analogs, (ii) ribose analogs, (iii) 2′-ribofluoro-2′-deoxyuridine analogs, and (iv) 2′,3′-dideoxyuridine analogs. Among the compounds **1**–**43** tested, nucleosides possessing 2′-deoxyribose moiety viz., 5-fluoro-2′-deoxyuridine (**1**), 5-chloro-2′-deoxyuridine (**2**), 5-(1-hydroxyethyl)-2′-deoxyuridine (**41**), 5-(1-fluoro-2-chloroethyl)-3′,5′-di-*O*-acetyl-2′-deoxyuridine (**42**), and 5-(1-fluoro-2-bromothyl)-3′,5′-di-*O*-acetyl-2′-deoxyuridine (**43**) were found to exhibit moderate to significant inhibitory activity against *M. bovis* and/or *M. avium* (Table 1). The most potent compounds of the 2′-deoxyuridine series **41**–**43** inhibited the growth of *M. avium* (MIC_{90} = 1–5 μg/mL range) at concentrations which compared favorably with that of reference drug rifampicin or clarithromycin (MIC_{90} = 1–2 μg/mL). Compounds **41**–**43** exhibited superior antimycobacterial activity compared to those of 5-fluoro-2′-deoxyuridine (**1**) and 5-chloro-2′-deoxyuridine (**2**), where **1** showed 80% inhibition at 50 μg/mL and **2** was not inhibitory to *M. avium* up to a concentration of 100 μg/mL. The antimycobacterial activity of **41**–**43** against *M. bovis* was significantly lower than that observed against *M. avium*. The 5-bromo- (**3**), 5-iodo- (**4**), 5-hydroxy- (**5**) and 5-hydroxymethyl- (**6**), 5-hydroxyethyl- (**7**), 5-methoxymethyl- (**8**), 5-ethyl- (**9**), 5-vinyl- (**10**), (*E*)-5-(2-chlorovinyl)- (**11**), and (*E*)-5-(2-bromovinyl)- (**12**) derivatives of 2′-deoxyuridine were devoid of antimycobacterial activity against both *M. bovis* and *M. avium*. These studies suggest that the hydroxyl and fluoro groups at the C-1 of the 5-ethyl side chain are important for potent antimycobacterial activity. In addition, C-1 substituents with or without the halogen atoms at the C-2 of the 5-side chain collectively influence the anti-mycobacterial activity. The 3′,5′-di-*O*-acetyl derivatives of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (**14**) and (*E*)-5-(2-iodovinyl)-2′-deoxyuridine (**15**) did not exhibit any anti-mycobacterial activity in contrast to the potent anti-mycobacterial activity of **42** and **43**. These results further suggest that 5-(C-1 substituted alkyl) side chains contribute to anti-mycobacterial activity in compounds **42** and **43**. Removal of acetyl groups from compounds **42** and **43** under acidic or basic conditions provided decomposed products. However, treatment with porcine liver esterase at 37 °C, followed by character-



Scheme 2. Reagents and conditions: (i) tritylchloride, pyridine, 45 °C; (ii) mesylchloride, pyridine, 25 °C; (iii) $(Bu)_4NF$, THF, 25 °C (method A); (iv) NaOH, C_2H_5OH, refluxing (method B); (v) methanesulfonylchloride, pyridine, 0 °C; (vi) NaOH, H_2O, reflux; (vii) *t*-BuOK, DMSO, 25 °C.

ization of the obtained compounds by HRMS, suggested that they could be converted to deacylated analogs in biological milieu.

The 5-halo- (**16**–**18**), 5-methyl-(**19**), 5-hydroxy-(**20**), and (*E*)-5-(2-bromovinyl)-(**21**) substituted ribose derivatives, as well as their 2′-deoxyribose analogs, were not inhibitory against *M. bovis* or *M. avium*. Similarly, 5-substituted 2′-ribofluoro-2′-deoxyuridine analogs (**23**–**25**, **27**) were inactive against both mycobacteria, except for 5-methyl-2′-ribofluoro-2′-deoxyuridine (**26**), which exhibited moderate inhibition of *M. bovis* multiplication at 50 μg/mL (50%).

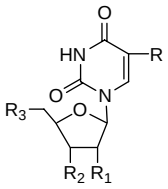
Within the 2′,3′-dideoxyuridine analogs, none of the 3′-azido derivatives viz., 3′-azido-2′,3′-dideoxyuridine (**28**) and 3′-azido-2′,3′-dideoxythymidine (**29**); 3′-fluoro analogs viz., 3′-fluoro-2′,3′-dideoxyuridine (**30**), 3′-fluoro-2′,3′-dideoxythymidine (**31**), and 5-halo-3′-fluoro-2′,3′-dideoxyuridine (**32**–**34**); 2′,3′-dideoxy derivatives viz., 2′,3′-dideoxyuridine (**35**), 5′-*O*-acetyl-2′,3′-dideoxyuridine (**36**), 2′,3′-dideoxythymidine (**37**), and 5-iodo-2′,3′-dideoxyuridine (**38**) showed inhibitory effect against both *M. bovis* and *M. avium*. However, 2′,3′-didehydro-2′,3′-dideoxythymidine (**39**) exhibited moderate activity only against *M. avium* at 100 (50%) and 50 (25%) μg/mL concentrations, whereas related 5-iodo-2′,3′-didehydro-2′,3′-dideoxyuridine (**40**) was devoid of activity.

The complete genome sequence of *M. tuberculosis* has been deciphered.[29] It encodes many of the enzymes required for DNA and RNA synthesis, and pyrimidine and purine nucleoside biosynthesis. Thymidine monophosphate kinase from *M. tuberculosis* (TMPKmt) has recently been investigated as a new target for the drug design against mycobacteria.[30] It plays a significant role in DNA synthesis, lies in the junction of salvage and de novo pathway of pyrimidine biosynthesis, and is only 22% homologous to human TMP kinase. TMPKmt phosphorylates thymidine monophosphate to the thymidine diphosphate. However, it has been shown that this enzyme has high affinity for thymidine and other nucleosides as well.[31] In the search for selective inhibitors of TMPKmt, several base and sugar modified pyrimidine nucleoside analogs have been investigated.[30–33] Among the compounds reported here, 5-chloro-2′-deoxyuridine (**2**), 5-bromo-2′-deoxyuridine (**3**), 5-iodo-2′-deoxyuridine (**4**), 3′-azido-2′,3′-dideoxythymidine (**29**), and 3′-fluoro-2′,3′-dideoxythymidine (**30**) were identified as the most potent competitive inhibitors of TMPKmt with low K_i values[30–33]. However, in whole cell-based assays, these pyrimidine nucleosides did not exhibit any anti-mycobacterial activity, except for **2** that showed 80% inhibition at 50 μg/mL. In contrast, compounds **1** and **26** with a higher K_i value for TMPKmt also exhibited moderate activity against *M. bovis* (50% at 50 μg/mL). Thus, the ability of a compound to function as a selective inhibitor of TMPKmt and its antimycobacterial properties may not correlate well and may be confounded by the cell entry and metabolism.

We examined the antimycobacterial activity of test nucleosides by direct mycobacterial growth inhibition since a cell-based assay would include the steps of entry into bacterial cells and metabolism, which could otherwise limit their efficacy. It is envisaged that several enzymes involved in nucleic acid metabolism could be targeted by novel anti-tuberculosis agents. The exact mechanism of action of the compounds inhibiting mycobacterial multiplication in this study is not yet known. However, it is possible that active compounds after their metabolic conversion to phosphorylated forms by mycobacterial kinases may be selectively inhibiting its DNA and/or RNA synthesis, by acting as substrates and/or inhibitors of metabolic enzymes of DNA/RNA synthesis.

The compounds showing significant activity were examined for their toxicity in vitro in monkey kidney (Vero cells) and human foreskin fibroblast (HFF cells)

Table 1. In vitro antimycobacterial activity of test compounds against *Mycobacterium bovis* and *Mycobacterium avium*



Compound	R	R_1	R_2	R_3	Antimicrobial activity[a] % inhibition (concentration μg/mL)	
					M. bovis	*M. avium*
1	F	H	OH	OH	50 (50)	80 (50)
2	Cl	H	OH	OH	80 (50) 25 (10)	0
3	Br	H	OH	OH	0	0
4	I	H	OH	OH	0	0
5	OH	H	OH	OH	0	0
6	CH_2OH	H	OH	OH	0	0
7	CH_2CH_2OH	H	OH	OH	0	0
8	CH_2OCH_3	H	OH	OH	0	0
9	C_2H_5	H	OH	OH	0	0
10	CH=CH_2	H	OH	OH	0	0
11	CH=CHCl	H	OH	OH	0	0
12	CH=CHBr	H	OH	OH	0	0
13	CH=CHI	H	OH	OH	0	0
14	CH=CHBr	H	OAc	OAc	0	0
15	CH=CHI	H	OAc	OAc	0	0
16	F	OH	OH	OH	10 (50)	0
17	Br	OH	OH	OH	0	0
18	I	OH	OH	OH	0	0
19	CH_3	OH	OH	OH	0	0
20	OH	OH	OH	OH	0	0
21	CH=CHBr	OH	OH	OH	0	0
22	CH_3	OCH_3	OH	OH	0	0
23	H	F	OH	OH	0	0
24	F	F	OH	OH	0	0
25	I	F	OH	OH	0	0
26	CH_3	F	OH	OH	50 (50)	0
27	CH=CHI	F	OH	OH	0	0
28	H	H	N_3	OH	0	0
29	CH_3	H	N_3	OH	0	0
30	H	H	F	OH	0	0
31	CH_3	H	F	OH	0	0
32	Cl	H	F	OH	0	0
33[d]	Br	H	F	OH	0	0
34[d]	F	H	F	OH	0	0
35	H	H	H	OH	0	0
36	H	H	H	OAc	0	0
37	CH_3	H	H	OH	0	0
38	I	H	H	OH	0	0
39	CH_3	Double bond		OH	0	50 (100); 25 (50)
40	I	Double bond		OH	0	0
41	$CH(OH)CH_3$	H	OH	OH	75 (100); 0 (50)	90 (5)[b]
42	$CH(F)CH_2Cl$	H	OAc	OAc	50 (100); 0 (50)	90 (1–5)
43	$CH(F)CH_2Br$	H	OAc	OAc	25 (50)	90 (1–5)
Rifampicin[c]	—	—	—	—	100 (1)	90 (2)
Clarithromycin[c]	—	—	—	—	ND	95 (2)

[a] Antimycobacterial activity was determined at concentrations 100, 50, 10, and 1 μg/mL.
[b] Range from three independent repeated experiments.
[c] Positive control drugs.
[d] Antimycobacterial activity was determined at concentrations 50, 10, and 1 μg/mL.

cell lines up to 200 μg/mL concentration. The compounds **41**–**43** did not display toxicity against both HFF and Vero cells up to the highest concentrations tested (CC_{50} = >200 μg/mL), whereas compounds **1** and **2** showed significant toxicity (CC_{50} = <50 μg/mL).

4. Summary

Discovery of new lead compounds working by different mechanisms of action is urgently required for the treatment of mycobacterial infections due to the emerging global health threat of tuberculosis. We have identified 5-(1-substituted alkyl) pyrimidine nucleosides (**41–43**) as inhibitors of *M. avium* multiplication. Among a number of analogs investigated, it was observed that the presence of a 5-(1-substituted alkyl) chain at the 5-position of the pyrimidine base is an important determinant for specific antimycobacterial activity against *M. avium*. This new class of agents can be optimized further for the design and investigation of anti-tuberculosis agents. Further structure–activity relationship studies are ongoing on this class of compounds in our laboratories.

5. Experimental

Melting points were determined with a Buchi capillary apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were determined for solutions in Me_2SO-d_6, MeOH-d_4 or $CDCl_3$ on a Bruker AM 300 spectrometer using Me_4Si as an internal standard (^{1}H NMR). The assignment of all exchangeable protons (OH, NH) was confirmed by the addition of the D_2O. ^{13}C NMR spectra were acquired using the *J* modulated spin echo technique where methyl and methine carbon resonances appear as positive peaks, and methylene and quaternary carbons as negative peaks. Micro-analyses were within ±0.4% of theoretical values for all elements listed, unless otherwise indicated. Silica gel column chromatography was carried out using Merck 7734 silica gel (100–200 μM particle size). Thin layer chromatography (TLC) was performed with Whatman MK6F silica gel microslides (25 μM thickness). 5-Fluoro-2′-deoxyuridine (**1**), 5-ethyl-2′-deoxyuridine (**9**), 5-fluorouridine (**16**), 5-hydroxyuridine (**20**), and 3′-deoxythymidine (**37**) were purchased from Sigma–Aldrich. 5-Hydroxy-2′-deoxyuridine (**5**), 5-hydroxymethyl-2′-deoxyuridine (**6**), 5-hydroxyethyl-2′-deoxyuridine (**7**), 5-methoxymethyl-2′-deoxyuridine (**8**), and 5-methyluridine (**19**) were purchased from Berry & Associates.

5.1. (*E*)-5-(2-Chlorovinyl)-2′-deoxyuridine (11)

N-Chlorosuccinimide (21.36 mg, 0.16 mmol) was added slowly with stirring to a solution of **44** (50 mg, 0.16 mmol) in dioxane/water (3:7, v/v, 5 mL) and glacial acetic acid (15 μL), during a period of 5 min. The reaction was allowed to proceed for 2 h at 25 °C. An additional aliquot of *N*-chlorosuccinimide (11 mg, 0.082 mmol) was added to the reaction mixture and stirred at 25 °C overnight. Removal of the solvent in vacuo gave a residue, which was purified on a silica gel column using chloroform/methanol (90:10, v/v) as eluent to yield **11**, as a white solid after recrystallization from methanol (20 mg, 43%). The product was identical in all aspects with the compound previously reported.[16] mp 152–55 °C (dec.); ^{1}H NMR (DMSO-d_6) δ: 2.10 (m, 2H, H-2′), 3.60 (m, 2H, H-5′), 3.78 (m, 1H, H-4′), 4.23 (m, 1H, H-3′), 5.12 (t, 1H, 5′-OH), 5.28 (d, 1H, 3′-OH), 6.12 (t, 1H, J = 6 Hz, H-1′), 6.56 (d, J = 13.2 Hz, 1H, vinylic H), 7.15 (d, J = 13.2 Hz, 1H, vinylic H), 8.04 (s, 1H, H-6), 11.60 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 60.97 (C-5′), 69.92 (C-3′), 84.51 (C-1′), 87.51 (C-4′), 108.62 (C-5), 117.88 (C-8), 126.30 (C-7), 139.17 (C-6), 149.24 (C-2), 161.64 (C-4).

5.2. (*E*)-5-(2-Bromovinyl)-2′-deoxyuridine (12)

N-Bromosuccinimide (0.168 mmol, 30 mg) was added to a solution of **44** (50 mg, 0.167 mmol) in water (2 mL) and THF (3 mL), and the reaction was allowed to proceed at 25 °C for 30 min, at which time TLC indicated that the reaction was completed. Removal of the solvent gave a residue, which was purified by silica gel column chromatography. Elution with chloroform/methanol (90:10, v/v) afforded (**12**). The product (53 mg, 95% yield) was identical in all aspects with the compounds previously prepared.[15] mp 124–30 °C (dec.); ^{1}H NMR (DMSO-d_6) δ: 2.12 (m, 2H, H-2′), 3.6 (m, 2H, H-5′), 3.78 (m, 1H, H-4′), 4.23 (m, 1H, H-3′), 5.10 (t, J = 4.8 Hz, 1H, 5′-OH), 5.25 (d, 1H, J = 4.56 Hz, 1H, 3′-OH), 6.12 (t, 1H, J = 6.0 Hz, H-1′), 6.82 (d, J = 13.2 Hz, 1H, vinylic H), 7.24 (d, J = 13.2 Hz, 1H, vinylic H), 8.08 (s, 1H, H-6), 11.60 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 40.18 (C-2′), 61.30 (C-5′), 70.32 (C-3′), 85.12 (C-1′), 87.75 (C-4′), 107.30 (C-8), 110.27 (C-5), 130.04 (C-7), 139.77 (C-6), 149.72 (C-2), 162.14 (C-4).

5.3. 3′,5′-Di-*O*-acetyl-(*E*)-5-(2-bromovinyl)-2′-deoxyuridine (14)

(*E*)-5-(2-Bromovinyl)-2′-deoxyuridine (**12**, 180 mg, 0.54 mmol) was dissolved in a mixture of acetic anhydride (0.25 mL) and dry pyridine (10 mL). The reaction mixture was allowed to stand at room temperature for 24 h. Removal of the solvent in vacuo and crystallization of the product by chloroform/hexanes yielded **14** (200 mg, 99%). ^{1}H NMR (DMSO-d_6) δ: 2.12 and 2.14 (2s, 6H, $COCH_3$), 2.16 and 2.56 (2m, 2H, H-2′), 4.26–4.46 (m, 3H, H-4′, H-5′), 5.22 (m, 1H, H-3′), 6.28 (t, 1H, J = 6.0 Hz, H-1′), 6.68 (d, J = 13.0 Hz, 1H, vinylic H), 7.42 (d, J = 13.0 Hz, 1H, vinylic H), 7.50 (s, 1H, H-6), 8.62 (s, 1H, NH). Anal. Calcd for $C_{15}H_{17}BrN_2O_7$: C, 43.16; H, 4.07; N, 6.71. Found: C, 43.47; H, 4.35; N, 6.41.

5.4. 3′,5′-Di-*O*-acetyl-(*E*)-5-(2-iodovinyl)-2′-deoxyuridine (15)

Acetic anhydride (4 mL) was added to a solution of **44** (400 mg, 1.34 mmol) in pyridine (5 mL) with stirring and the reaction was allowed to proceed for 24 h at 25 °C. Removal of the solvent in vacuo and crystallization of the product by ethanol provided the di-*O*-acetylated derivative **45** that was reacted with *N*-iodosuccinimide (240 mg, 1.06 mmol) and potassium acetate (200 mg, 2.04 mmol) in dry DMF (10 mL) with stirring at room temperature for 24 h. Removal of the solvent and purification of the product by silica gel column chromatography using chloroform/acetone (90:10, v/v) as eluent

yielded **15** (340 mg, 55%) as a syrup. ^{1}H NMR (DMSO-d_6) δ: 2.10 and 2.12 (2s, 6H, $COCH_3$), 2.14 and 2.55 (2m, 2H, H-2′), 4.24–4.44 (m, 3H, H-4′, H-5′), 5.20 (m, 1H, H-3′), 6.25 (t, 1H, J = 6.0 Hz, H-1′), 7.0 (d, J = 13.0 Hz, 1H, vinylic H), 7.40 (d, J = 13.0 Hz, 1H, vinylic H), 7.48 (s, 1H, H-6), 9.54 (s, 1H, NH). Anal. Calcd for $C_{15}H_{17}IN_2O_7$: C, 38.79; H, 3.66; N, 6.03. Found: C, 38.51; H, 3.98; N, 6.32.

5.5. 5-Iodo-3′-*O*-mesyl-5′-*O*-trityl-2′-deoxyuridine (49)

A mixture of 5-iodo-2′-deoxyuridine (**4**) (1.3 g, 3.6 mmol) and trityl chloride (1.11 g, 4.0 mmol) in anhydrous pyridine (25 mL) was heated at 45 °C for 6 h and kept overnight at room temperature. The reaction was cooled to 0 °C, mesyl chloride (0.56 mL, 7.0 mmol) was added, and the reaction mixture was stirred for 90 min at room temperature. After the addition of H_2O (1 mL), the solvent was evaporated, and the resulting oil was diluted with chloroform (100 mL), washed with cold H_2O (2× 25 mL), dried (Na_2SO_4), and evaporated. The residue was purified by silica gel column chromatography by chloroform/methanol (97:3, v/v) as eluent to yield **49** (1.6 g, 66%) as foam. ^{1}H NMR ($CDCl_3$) δ: 2.47 (m, 1H, H-2′), 2.80 (m, 1H, H-2′), 3.06 (s, 3H, CH_3SO_2), 3.52 (m, 2H, H-5′), 4.38 (m, 1H, H-4′), 5.37 (m, 1H, H-3′), 6.35 (t, J = 6.0 Hz, 1H, H-1′), 7.28–7.50 (m, 15H, trityls), 8.16 (s, 1H, H-6), 9.23 (s, 1H, NH). Anal. Calcd for $C_{29}H_{27}IN_2O_7S$: C, 51.63; H, 4.00; N, 4.15. Found: C, 51.89; H, 4.27; N, 4.09.

5.6. Reaction of 5-iodo-3′-*O*-mesyl-5′-*O*-trityl- 2′-deoxyuridine (49) with tetra-*n*-butyl ammonium fluoride in tetrahydrofuran (method A)

A solution of **49** (0.21 g, 0.31 mmol) in THF (4 mL) containing 1 M TBAF was stored at room temperature for 24 h. After evaporation of the solvent, the reaction mixture was divided between $CHCl_3$ (25 mL) and H_2O (25 mL). The chloroform layer was separated, dried (Na_2SO_4) and evaporated. The residue obtained was purified by silica gel column chromatography using chloroform/methanol (98:2, v/v) as eluent to give 2,3′-anhydro-5-iodo-5′-*O*-trityl-2′deoxyuridine **51** (0.12 g, 67%). ^{1}H NMR (DMSO-d_6) δ: 2.56 (m, 2H, H-2′), 3.14 (m, 2H, H-5′), 4.46 (m, 1H, H-4′), 5.36 (m, 1H, H-3′), 5.98 (t, 1H, H-1′), 7.20–7.40 (m, 15H, trityls), 8.36 (s, 1H, H-6). Anal. Calcd for $C_{28}H_{23}IN_2O_4$: C, 58.13; H, 3.97; N, 4.84. Found: C, 58.49; H, 4.23; N, 5.13.

5.7. 2,3′-Anhydro-5-iodo-5′-*O*-trityl-2′deoxyuridine (51) (method B)

To a refluxing ethanolic solution of **49** (1 g, 1.48 mmol) was added a solution of 1 N NaOH (1.5 mL, 1.5 mmol) dropwise and the solution was refluxed for 30 min. Removal of the solvent and purification, as described for method A, provided **51** (0.7 g, 82%) as a syrup. The ^{1}H NMR and elemental analysis of **51** were identical to that of **51** reported in method A.

5.8. 3′,5′-Di-*O*-mesyl-5-iodo-2′-deoxyuridine (52)

5-Iodo-2′-deoxyuridine (**4**) (1.06 g, 3.0 mmol) in dry pyridine (10 mL) was added with methanesulfonylchloride (0.56 mL, 7.0 mmol), left at 0 °C for 12 h, and poured onto ice-water (50 mL) to give a solid precipitate, which was filtered. The wet solid was dissolved in chloroform and dried with sodium sulfate. Evaporation of the solvent gave a syrup, which was purified by silica gel column chromatography, using chloroform/methanol (93:7, v/v), to yield **52** as a syrup (1.3 g, 83.3%). ^{1}H NMR (DMSO-d_6) δ: 2.60 (m, 2H, H-2′), 3.34 (s, 3H, SO_2CH_3), 4.38 (m, 1H, H- 4′), 4.50 (m, 2H, H-5′), 5.30 (m, 1H, H-3′), 6.14 (t, J = 6.0 Hz, 1H, H-1′), 8.10 (s, 1H, H-6), 11.80 (s, 1H, NH). Anal. Calcd for $C_{11}H_{15}IN_2O_9S_2$: C, 25.88; H, 2.94; N, 5.49. Found: C, 26.10; H, 3.26; N, 5.24.

5.9. 3′,5′-Anhydro-5-iodo-2′-deoxyuridine (53)

5-Iodo-3′,5′-di-*O*-mesyl-2′-deoxyuridine **52** (1.02 g, 2.0 mmol) was added in portions to a stirred solution of sodium hydroxide (0.24 g, 6.0 mmol) in water (5 mL) whereupon the reaction mixture became yellow-orange. The reaction mixture was then heated to reflux for 45 min. After cooling the reaction mixture to 25 °C, concd HCl (0.17 mL) was added. The resulting solution was concentrated under vacuo on a rotary evaporator and filtered. The white solid obtained was dissolved in methanol and purified by silica gel column chromatography using chloroform/methanol (95:5, v/v) as eluent to yield **53** as a syrup (0.2 g, 30%). ^{1}H NMR (DMSO-d_6) δ: 2.54 (m, 2H, H-2′), 3.96 (d, J = 8.0 Hz, 1H, H-4′), 4.72 (dd, J = 8.0 Hz, 1H, H-5′), 4.91 (m, 2H, H-5′), 5.50 (m, 1H, H-3′), 6.49 (d, J = 6.0 Hz, 1H, H-1′), 8.62 (s, 1H, H-6), 11.78 (s, 1H, NH). Anal. Calcd for $C_9H_9IN_2O_4$: C, 32.14; H, 2.67; N, 8.33. Found: C, 32.28; H, 3.00; N, 8.51.

5.10. 2′,3′-Didehydro-2′,3′-dideoxy-5-iodouridine (40)

Potassium *tert*-butoxide (0.27 g, 2.4 mmol) was added to a solution of oxetane **53** (0.4 g, 1.2 mmol) in dry DMSO (7.5 mL) at 10–15 °C under nitrogen. After the addition was complete, the reaction mixture was stirred for 2 h at 25 °C. The reaction mixture was neutralized to pH ~7 by acetic acid and the solvent was removed on rotavapor in vacuo. The collected yellowish oily residue was triturated two-three times with hot acetone. The acetone washings evaporated to dryness on a rotavapor were dissolved in ethanol and decolorized by charcoal, filtered and evaporated on the rotavapor. The obtained residue was purified by silica gel column chromatography using chloroform/methanol (95:5,v/v) as eluent to yield **40** (0.3 g, 75%). ^{1}H NMR (DMSO-d_6) δ: 3.62 (dd, J = 4.8, 3.6 Hz, 2H, H-5′), 4.82 (s, 1H, H- 4′), 5.12 (t, J = 4.0 Hz, 1H, 5′-OH), 5.92 (dd, J = 1.2, 5.0 Hz, 1H, H-2′), 6.40 (d, 1H, J = 6.0 Hz, H-3′), 6.80 (d, J = 1.2 Hz, 1H, H-1′), 8.32 (s, 1H, H-6), 11.64 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ: 61.61 (C-5′), 68.81 (C-5), 87.69 (C-4′), 89.41 (C-1′), 125.88 (C-2′), 135.56 (C-3′), 146.13 (C-6), 150.53 (C-2), 160.58 (C-4). Anal. Calcd for $C_9H_9IN_2O_4$: C, 32.14; H, 2.67; N, 8.33. Found: C, 31.88; H, 2.43; N, 8.16.

5.11. In vitro antimycobacterial activity assay (*M. bovis, M. avium*)

Mycobacterium bovis (BCG), *M. avium* (ATCC 25291) were obtained from the American Type Culture Collection, Rockville, MD. The antimycobacterial activity was determined using microplate alamar blue assay (MABA).[28] Test compounds were dissolved in DMSO at 100× of the highest final concentration used and subsequent dilutions were performed in 7H9GC (Difco Laboratories, Detroit, Michigan) media in 96-well plates. For these experiments, each compound was tested at 100, 50, 10, and 1 μg/mL in triplicate. The experiments were repeated three times and the mean percent inhibition is reported in the table. The standard deviations were within 10%. Frozen mycobacterial inocula were diluted in media 7H9GC and added to each well at 2.5×10^5 CFU/mL final concentration. Sixteen control wells consisted of 8 with bacteria alone (B) and 8 with media alone (M). Plates were incubated for an initial 6 days and starting from 6 days of incubation, 20 μL of 10× alamar blue and 12.5 μL of 20% Tween 80 were added to one M and one B well. Wells were observed for 24–48 h for visual color change from blue to pink and read by a spectrophotometer (at excitation 530/525 and emission 590/535) to determine the OD values. If the B well became pink by 24 h (indicating growth), the reagent was added to the entire plate. If the B well remained blue, additional M and B wells were tested daily until bacterial growth could be visualized by color change. After the addition of the reagent to the plate, cultures were incubated for 24 h and plates were observed visually for color change and also read by a spectrophotometer. Visual MIC was defined as the lowest concentration of a compound that prevented a color change from blue to pink. Percent inhibition was calculated as (test well-M bkg./B well-M bkg.) × 100. The lowest drug concentration effecting an inhibition of ~90% was considered as the MIC_{90}. Similar methodology was used for both *M. bovis* BCG and *M. avium*. Rifampicin and clarithromycin were used as positive controls. As negative controls, DMSO was added to the B well at a concentration similar to that of compound wells, M wells served as negative controls. In most of the experiments, the M wells gave OD of 3000–4000, and the B wells had OD values were 60,000–1,00,000.

5.12. Cell cytotoxicity assay

Cell viability was measured using the cell proliferation kit 1 (MTT; Boehringer Mannheim), as per manufacturer's instructions. Briefly, a 96-well plate was seeded with Vero cells or HFF cells at a density of 2.5×10^5 cells per well. Cells were allowed to attach for 6–8 h, the media were replaced with media containing drugs at concentrations of 200, 100, 50, 25, 12.5, 6.3, and 1.5 μg/mL. DMSO was also included as control. Plates were incubated for 3 days at 37 °C. The color reaction involved adding 10 μL MTT reagent per well, incubating 4 h at 37 °C, and then adding 100 μL solubilization reagent. Plates were read on an ELISA plate reader (Abs 560 nm), following an overnight incubation at 37 °C.

Acknowledgments

We are grateful to the Canadian Institutes of Health Research (CIHR) for an operating grant (MOP-49415) (R.K. and D.Y.K.) for the financial support of this research.

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