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Inhibition of Mycobacterial Replication by Pyrimidines Possessing Various C-5 Functionalities and Related 2′-Deoxynucleoside Analogues Using in Vitro and in Vivo Models

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Tuberculosis (TB) has become an increasing problem since the emergence of human immunodeficiency virus and increasing appearance of drug-resistant strains. There is an urgent need to advance our knowledge and discover a new class of agents that are distinct than current therapies. Antimycobacterial activities of several 5-alkyl, 5-alkynyl, furanopyrimidines and related 2′-deoxynucleosides were investigated against *Mycobacterium tuberculosis*. Compounds with 5-arylalkynyl substituents (**23**−**26**, **33**, **35**) displayed potent in vitro antitubercular activity against *Mycobacterium bovis* and *Mycobacterium tuberculosis*. The in vivo activity of 5-(2-pyridylethynyl)-uracil (**26**) and its 2′-deoxycytidine analogue, 5-(2-pyridylethynyl)-2′-deoxycytidine (**35**), was assessed in BALB/c mice infected with *M. tuberculosis* (H37Ra). Both compounds **26** and **35** given at a dose of 50 mg/kg for 5 weeks showed promising in vivo efficacy in a mouse model, with the 2′-deoxycytidine derivative being more effective than the uracil analogue and a reference drug D-cycloserine. These data indicated that there is a significant potential in this class of compounds.

Introduction

Tuberculosis (TB[a]) is an ancient infectious disease that still remains a leading cause of morbidity and mortality worldwide. At the present time, nearly two billion people worldwide are infected with the tubercle bacillus, and the prevalence of active TB is increasing.[1−3] The latent infection in many of these individuals may reactivate sometime later in life. Tubercle bacilli can be contained in the presence of effective cellular immunity but immunocompromised status such as human immunodeficiency virus (HIV) infection, cancer chemotherapy, or use of immunosuppressive drugs in transplantation provides the single most significant factor in reactivation of the latent TB leading to full clinical disease.[4,5]

TB and HIV have formed a new and deadly combination. There is a resurgence in the incidence of TB with high rates of HIV−TB coinfections. *Mycobacterium tuberculosis* (*M. tuberculosis*) poses a significant challenge to the clinical management of TB in HIV-infected patients and is often responsible for their death.[6] Bacillus Calmette Guerin (BCG)[6] is an attenuated strain of *Mycobacterium bovis* that is >98% homologous to *M. tuberculosis* and, therefore, is closely related to *M. tuberculosis*. Interestingly, *M. bovis* infections have re-emerged and are causing TB in humans, particularly those who are HIV positive.

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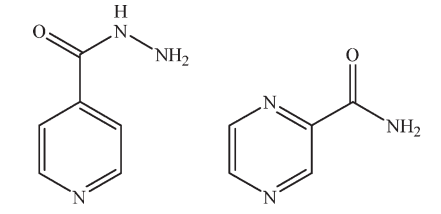
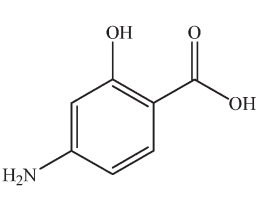
[a] Abbreviations: TB, tuberculosis; *M. bovis*, *Mycobacterium bovis*; *M. tuberculosis*, *Mycobacterium tuberculosis*; *M. avium*, *Mycobacterium avium*; BCG, Bacillus Calmette Guerin; MABA, microplate alamar blue assay; CFU, colony forming unit; *B. subtilis*, *Bacillus subtilis*; *S. aureus*, *Staphylococcus aureus*; *S. pneumoniae*, *Streptococcus pneumoniae*; *S. pyogenes*, *Streptococcus pyogenes*; SAR, structure−activity relationship; INH, Isoniazid.

Chemotherapy with antituberculosis drugs such as streptomycin, rifampicin and several six-membered aromatic ring derivatives such as isoniazid (**1**), pyrazinamide (**2**), *p*-aminosalicylic acid (**3**), and ethionamide (**4**) revolutionized TB therapy in the 1970s and resulted in a rapid decline in tuberculosis in many developed countries. However, there is now an ever-increasing threat of drug-resistant TB becoming epidemic in many countries because no new classes of TB-specific drugs have been developed since rifampicin.[2,3,7−9]

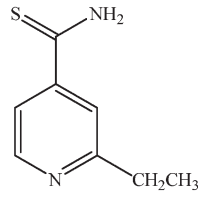


1 Isoniazid

2 Pyrazinamide



3 p-Aminosalicylic acid



4 Ethionamide

Drug resistance has been observed for all of the first-line antimycobacterial drugs as well as for several second-line

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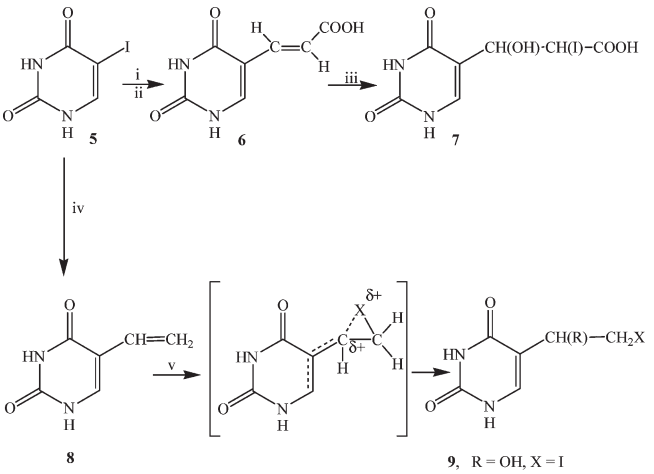
antituberculosis drugs.[10] Although drug-resistant TB was reported before the emergence of HIV, the problem of drug-resistant TB was not as severe as it is today.[6,10,11] Thus, there exists an urgent need for the design of new classes of antimycobacterial agents which the bacteria have never encountered.

In our earlier studies, we synthesized and evaluated various nucleoside compounds for their anti-TB activity.[12−17] Among different substituents, we revealed that pyrimidine nucleosides with acetylenic side chains at the C5 position exert remarkable in vitro inhibition of *M. bovis*, *M. avium*, and *M. tuberculosis*.[12,14,15] In these studies, we also noted that 5-(1-azido (or halo)-2-haloethyl) substituents and 5-alkylalkynyl moieties at the C-5 position of the substituted pyrimidines contributed to significant antimycobacterial activities.[13,16] As part of our ongoing program to identify agents that display potent anti-TB activity and to further explore the SARs, we have now designed, synthesized, and evaluated a new series of 5-alkyl (**7**, **9**−**16**) and 5-alkyl (or aryl)alkynyl pyrimidines (**17**−**26**, **28**−**31**) for their antimycobacterial properties. In these studies, we discovered that pyrimidines with 5-arylalkynyl pharmacophores (**23**−**26**) exhibit potent inhibition of *M. bovis* and *M. tuberculosis* in comparison to their 5-alkyl (**7**, **9**−**16**) and 5-alkylalkynyl (**17**−**22**) counterparts. Among the 5-arylalkynyl pyrimidine compounds, 5-(2-pyridylethynyl)-uracil (**26**) demonstrated most potent activity against *M. tuberculosis* (H37Ra) and was tested for its efficacy in a murine model of H37Ra infection. In our previous investigations, we observed that in order to confer a substantial antimycobacterial effect, a glycosyl moiety at the N-1 position of substituted uracil plays a crucial role.[12,14,15] Further, an amino group at C-4 appears to be preferred for improved anti-TB activity in the 5-alkynyl-2′-deoxyuridine series of compounds.[12] Encouraged by considerable in vivo inhibition of mycobacteria by compound **26**, we also synthesized 2′-deoxyuridine (**33**) and 2′-deoxycytidine (**35**) derivatives of 5-(2-pyridylethynyl)-uracil (**26**) and investigated their potential as anti-TB agents. Although both nucleosides 5-(2-pyridylethynyl)-2′-deoxyuridine (**33**) and 5-(2-pyridylethynyl)-2′-deoxycytidine (**35**) exhibited potent in vitro activity against *M. tuberculosis* (H37Ra) that were comparable, compound **35** demonstrated no cytotoxicity, therefore, it was selected for in vivo activity studies. Intriguingly, compound **35** substantially reduced the bacterial load in lung, liver, and spleen tissues in three out of five mice as compared to its uracil analogue (**26**). For the first time, we report the antimycobacterial potential of 5-alkynyl analogues of pyrimidine nucleosides in vivo.

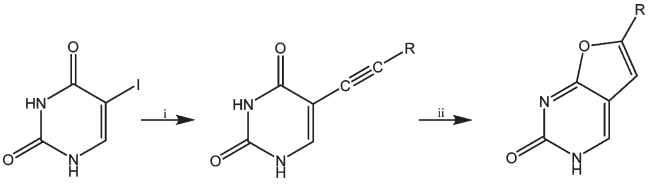
Scheme 1[a]



9, R = OH, X = I
10, R = OCH_3, X = Br
11, R = OCH_3, X = Cl
12, R = OCH_3, X = I
13, R = OEt, X = I
14, R = OCH_2CH_2Br, X = I
15, R = OCH_2CH_2F, X = I
16, R = OCH_2CF_3, X = I

[a] Reagents and conditions: (i) CH_2=CHCOOEt, $(Ph_3P)_2PdCl_2$, Et_3N; (ii) 0.5 N KOH (**6**); (iii) I_2, KIO_3, H_2O, H_2SO_4 (**7**); (iv) vinyl acetate, $(Ph_3P)_2PdCl_2$, Et_3N, 80 °C (**8**); (v) *N*-iodosuccinimide, H_2O, 25 °C (**9**), *N*-halosuccinimide, MeOH, 25 °C (**10**−**12**), *N*-iodosuccinimide, EtOH, 25 °C (**13**), iodine mono chloride, $BrCH_2CH_2OH$ (**14**), iodine mono chloride, FCH_2CH_2OH (**15**), iodine mono chloride, CF_3CH_2OH (**16**), 25 °C.

Scheme 2[a]



17, R = $(CH_2)_2CH_3$
18, R = $(CH_2)_4CH_3$
19, R = $(CH_2)_7CH_3$
20, R = $(CH_2)_9CH_3$
21, R = $(CH_2)_{10}CH_3$
22, R = $(CH_2)_{11}CH_3$
23, R = C_6H_5
24, R = C_6H_4-*n*-propyl
25, R = C_6H_4-*n*-pentyl
26, R = 2-pyridyl

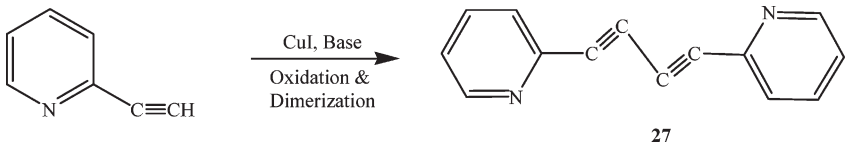
28, R = $(CH_2)_2CH_3$
29, R = $(CH_2)_4CH_3$
30, R = $(CH_2)_7CH_3$
31, R = $(CH_2)_9CH_3$

[a] Reagents and conditions: (i) $Pd(PPh_3)_4$, CuI, $(i\text{-}Pr)_2EtN$, DMF, N_2 atmosphere, room temperature, 1-pentyne (**17**), 1-heptyne (**18**), 1-decyne (**19**), 1-dodecyne (**20**), 1-tridecyne (**21**), 1-tetradecyne (**22**), phenylacetylene (**23**), 4-*n*-propylphenylacetylene (**24**), 1-ethynyl-4-pentylbenzene (**25**), 2-ethynylpyridine (**26**); (ii) CuI, Et_3N, dry MeOH, reflux.

Chemistry

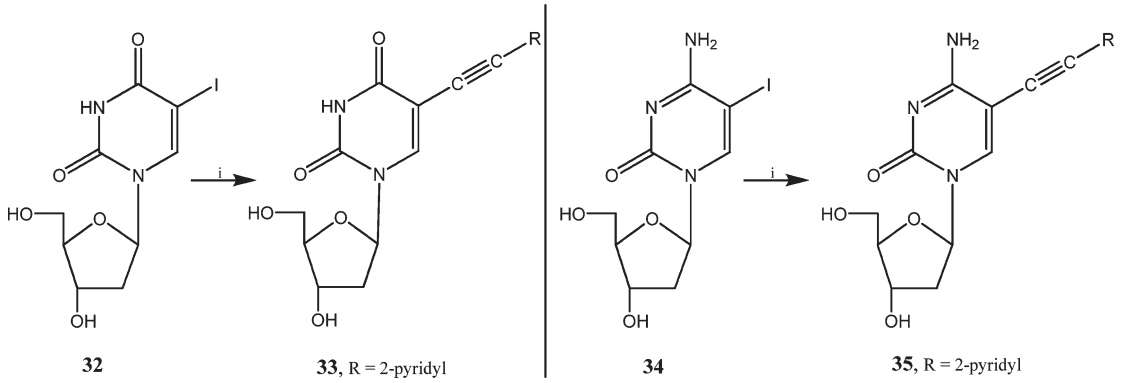
Synthesis of 5-alkyl uracils (**9**−**16**) (Scheme 1) was carried out using reported procedures described by us earlier.[18] 5-Alkynyl pyrimidines (**17**−**22**, **24**, **25**) were prepared by the Pd-catalyzed coupling reaction of 5-iodoracil (**5**) with appropriate alkylacetylenes, arylacetylenes, and para substituted arylacetylenes to yield the target 5-alkynyl pyrimidines **17**−**22**, **24**, **25** as described in Scheme 2. The 5-(2-phenylethynyl)-uracil (**23**) was synthesized using a previously reported method.[19] Our initial attempts to synthesize related 5-(2-pyridylethynyl)-uracil (**26**) were unsuccessful using similar reaction conditions

Scheme 3



27

Scheme 4[a]



[a] Reagents and conditions: (i) $Pd(PPh_3)_4$, CuI, $(i\text{-Pr})_2EtN$, 2-ethynylpyridine, DMF, N_2 atmosphere, room temperature.

where an undesired dimer of 2-ethynylpyridine was obtained in 30% yield (Scheme 3). The formation of the dimer, 1,4-bis-(2-pyridyl)-buta-1,3-diyne (**27**), can be explained based on oxidative homocoupling reactions[20] as depicted in Scheme 3. The structure of **27** was confirmed with the help of ^{1}H NMR, ^{13}C NMR spectroscopic data and microanalysis studies. To avoid unexpected dimerization mentioned above, we carried out the synthesis of compound **26** by taking extra precautions and completely eliminating moisture and oxygen from the reaction medium. The reaction flask was oven-dried and cooled in a nitrogen atmosphere and the reaction performed under positive pressure of nitrogen, yielding the desired compound **26** in 87% yield.

Cyclization[21] of selective 5-alkynyl uracils (**17**−**20**) in the presence of copper iodide and triethylamine in dry methanol at reflux temperature yielded the corresponding bicyclic furanopyrimidines (**28**−**31**) in 38−64% yields (Scheme 2).

Synthesis of 5-(2-pyridylethynyl)-2′-deoxyuridine (**33**) has been described by Pichat and co-workers[22] by the coupling reaction of a protected 3′,5′-trimethylsilylated 5-iodo-2′-deoxyuridine with alkynylzinc reagent in 17% overall yield. For the synthesis of nucleoside **33** and its 2′-deoxycytidine derivative (**35**), we used methodologies similar to that of compounds **17**−**25**. The single step Pd-catalyzed coupling reaction of 2-ethynylpyridine with respective 5-iodo-2′-deoxy nucleosides (**32**, **34**) provided target compounds **33** and **35** in excellent yields of 97% and 81%, respectively (Scheme 4).

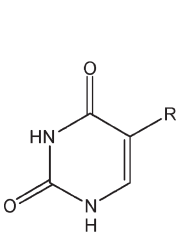
Results and Discussion

5-Substituted pyrimidines (**7**, **9**−**31**) and 2′-deoxy nucleosides (**33**, **35**) were evaluated in vitro against *M. bovis* and *M. tuberculosis* (H37Ra) by the microplate alamar blue assay (MABA) at 1−100 μg/mL concentrations. Rifampicin, isoniazid (INH), and D-cycloserine were used as positive control drugs. The results are summarized in Table 1. The 5-(1-hydroxy (or alkoxy)-2-haloethyl) pyrimidines (**7**, **9**−**16**, data not shown) did not prove to be inhibitory against both mycobacteria tested. In earlier studies,[16] we observed that in addition to 5-alkyl side chains, 5-alkylalkynyl (5-decynyl and 5-dodecynyl) moieties at the C-5 position of the specific N-1 substituted pyrimidines also contribute to significant antimycobacterial activities. Therefore, to further establish the structural requirements at the 5-position of pyrimidines for antimycobacterial activity, we explored various 5-alkyl (or aryl)alkynyl pyrimidines (**17**−**26**). Within these, 5-alkylalkynyl substituted pyrimidines (**17**−**22**, Table 1) were found to be inactive as antimycobacterial agents despite their varying chain lengths at the C-5 position. Together, these results suggest that in addition to the 5-substituent, N-1 glycosyl moieties also play a role in the antimycobacterial activity.

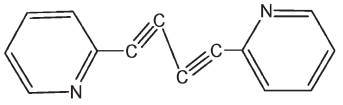
Encouragingly, among the pyrimidines (**17**−**26**), 5-arylalkynyl uracil derivatives **23**−**26** emerged as potent inhibitors of *M. bovis* and *M. tuberculosis*. 5-(4-*n*-Propylphenylethynyl)-uracil (**24**), 5-(4-*n*-pentylphenylethynyl)-uracil (**25**), and 5-(2-pyridylethynyl)-uracil (**26**) provided significant inhibition of both mycobacteria tested, *M. bovis* (MIC_{50} = 1−5 μg/mL) and *M. tuberculosis* (MIC_{50} = 10, 10, and 5 μg/mL, respectively), where compound **26** showed the best activity against H37Ra (Table 1). Among compounds **23**−**26**, it appears that the alkyl substituent present at the 4-position of the phenylalkynyl group (as in **24**, **25**) is influential for increased antimycobacterial effect because the compound **23** showed reduced inhibition of both *M. bovis* and *M. tuberculosis*. Replacement of the phenyl ring by a pyridinyl moiety (as in **26**) in the 5-alkynyl side chain led to significantly improved activity against both mycobacteria. The increased activity of **26** could possibly be due to increased interaction (H-bonding) of the heteroaryl ring with mycobacterial enzymes. In comparing the antimycobacterial activities of the arylalkynyl pyrimidines (**24**, **25**) with their corresponding inactive 5-alkylalkynyl derivatives (**17**, **18**), we note that inclusion of a phenyl ring completely reversed the activity of these molecules. These studies reflect that aromatic acetylene moieties at the 5-position of pyrimidine are crucial to provide antimycobacterial activities and conformational freedom of the alkyl side chain is not tolerated in this series of compounds. Alternatively, antimycobacterial action of compounds **23**−**26** could be due to the shape and size of the side chains that may produce steric hindrance. In contrast, inclusion of the aryl or *p*-alkylaryl ring in the 5-alkynyl side chain of the deoxy, dideoxy, and arabinouridine compounds was detrimental to antimycobacterial activities.[12,14,15] These studies suggest that pyrimidines **23**−**26** are perhaps working by different mechanism of action or targets than nucleoside compounds. The antimycobacterial activity of the dimer **27** was also evaluated. Surprisingly, compound **27** showed remarkable inhibition of BCG replication (80% at 0.5−1 μg/mL) and H37Ra replication (80% at 1 μg/mL). However, it was found to be cytotoxic [in the XTT cell cytotoxicity assay against a human hepatoma cell line (Huh-7 cells) CC_{50} = 10 μg/mL] in comparison to compounds **23**−**26** (CC_{50} = >100 μg/mL).

Modification of the 5-substituents in the pyrimidines **17**−**20** to the corresponding bicyclic analogues **28**−**31** yielded compounds with almost no antimycobacterial activity with

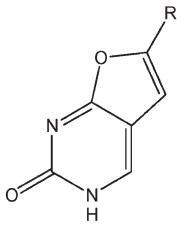
Table 1. In Vitro Antimycobacterial Activity of 5-Substituted Pyrimidines against *M. bovis* and *M. tuberculosis*[a]



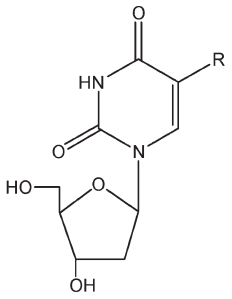
17-26



27



28-31



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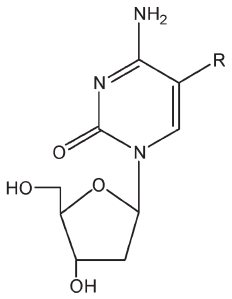

35

compd	R	*M. bovis* (BCG) % inhibition (concentration μg/mL)	MIC_{50} [μg/mL]	*M. tuberculosis* (H37Ra) % inhibition (concentration μg/mL)	MIC_{50} [μg/mL]
17	C≡C-$(CH_2)_2CH_3$	0		0	
18	C≡C-$(CH_2)_4CH_3$	0		0	
19	C≡C-$(CH_2)_7CH_3$	0		0	
20	C≡C-$(CH_2)_9CH_3$	0		0	
21	C≡C-$(CH_2)_{10}CH_3$	0		0	
22	C≡C-$(CH_2)_{11}CH_3$	0		0	
23	C≡C-C_6H_5	90 (100, 50), 40 (10)	[>10]	90 (100, 50), 40 (10)	[>10]
24	C≡C-C_6H_4-*n*-propyl	100 (100), 70 (50,10), 50 (1−5)	[1−5]	90 (100), 55 (50,10), 0 (5)	[<10]
25	C≡C-C_6H_4-*n*-pentyl	90 (100), 60 (50,10), 50 (1−5)	[1−5]	90 (100), 50 (50,10), 0 (5)	[10]
26	C≡C-(2-pyridyl)	90 (100), 75 (50,10), 60 (5)	[<5]	90 (100), 70 (50), 60 (10), 50 (5)	[5]
27		100 (100,50,10), 80 (0.5−1)	[<0.5]	100 (100,50,10), 80 (1)	[<1]
28	$(CH_2)_2CH_3$	90 (100), 50 (50,10)	[10−50]	50 (100), 0 (10)	[100]
29	$(CH_2)_4CH_3$	50 (100)	[100]	0	
30	$(CH_2)_7CH_3$	0		0	
31	$(CH_2)_9CH_3$	0		0	
33	C≡C-(2-pyridyl)	90 (100, 50), 80 (10), 50 (1−5)	[1−5]	90 (100), 78 (50), 68 (10), 50 (2.5−5)	[2.5−5]
35	C≡C-(2-pyridyl)	90 (100, 50), 80 (10), 50 (1−5)	[1−5]	90 (100, 50), 70 (10), 50 (2.5−5)	[2.5−5]
rifampicin[b]		100 (1)	[<1]	100 (1)	[<1]
INH[b,c]		100 (1)	[<1]	100 (1)	[<1]
cycloserine[b]		71 (10), 50 (5)	[5]	67 (10), 50 (5)	[5]

[a] Antimycobacterial activity was determined at concentrations 100, 50, 10, 5, 2.5, and 1 μg/mL. [b] Positive control drug. [c] INH = isoniazid.

the exception of a significant shift in the antimycobacterial activity of compound **28** (MIC_{50} = 10 μg/mL, BCG; 100 μg/mL H37Ra) when compared to its acyclic analogue **17**.

Subsequently, we tested compound **26** for its efficacy in female BALB/c mice infected with H37Ra at a dose of 50 mg/kg by the intraperitoneal route as described in detail in the Experimental Section. The mice were infected with *M. tuberculosis* strain H37Ra. Encouragingly, compound **26**, caused considerable reduction of the CFU counts in the lungs, liver, and spleen of the drug treated animals compared with those of the untreated controls (Figure 1A). Out of the five mice, compound **26** decreased the bacterial load in the lungs and liver of three mice (lungs: 75% inhibition in one, 50% inhibition in two; liver: 50% inhibition in three mice), but in the spleen only one mouse (70% inhibition) showed reduction in bacterial growth. Reduction in CFUs in lungs by compound **26** was statistically significant (P = 0.03) compared to untreated mice. However, the reduction in CFU counts in liver and spleen by compound **26** showed a trend but did not meet significance (P > 0.05). Compound **26** was found to be much less active than isoniazid administered at 25 mg/kg dose. However, no toxicity was observed in any of the mice treated with **26**.

In our previous investigations, we observed that in order to confer a substantial antimycobacterial effect, a glycosyl moiety at the N-1 position of substituted uracil plays a crucial role. Further, an amino group at C-4 appears to be preferred for improved anti-TB activity.[12,14,15] Upon the basis of these observations, we investigated the antimycobacterial activity of nucleoside derivatives of the most effective compound, **26**. Interestingly, both of the 2′-deoxy pyrimidine nucleoside analogues (**33**, **35**) showed potent inhibition of *M. bovis* and *M. tuberculosis* (MIC_{50} = 1−5, 2.5−5 μg/mL, respectively). Encouragingly, antimycobacterial activity of **33** and **35** against *M. tuberculosis* was also improved (68−70% inhibition at 10 μg/mL) as compared to their parent pyrimidine compound (**26**, 70% inhibition at 50 μg/mL). From these results, it is clear that 2-pyridylethynyl functionality at the C-5 position has the ability to retain the antimycobacterial activity in case of both pyrimidine and pyrimidine nucleosides. Although compounds **33** and **35** were found to exert comparable in vitro activity against H37Ra, the 2′-deoxycytidine analogue **35** showed no cytotoxicity in XTT and ^3H incorporation assays in Huh-7 cells up to a concentration of >200 μg/mL, whereas compound **33** showed 25% inhibition in cell viability at 200 μg/mL in the XTT assay. Therefore, compound **35** was selected to test its potency in the mouse model (H37Ra). Drug treatment was started five days post infection and was given for 5 weeks (5 days a week). Mice were treated with an intraperitoneal dose of compound **35** at 50 mg/kg formulated in 10% DMSO−saline. The efficacy of **35** was compared to that of D-cycloserine at 50 mg/kg and isoniazid at 25 mg/kg, which were administered as parallel treatment. The results are described in Figure 1B. Compound **35** showed significant activity in three out of five mice (85−90%

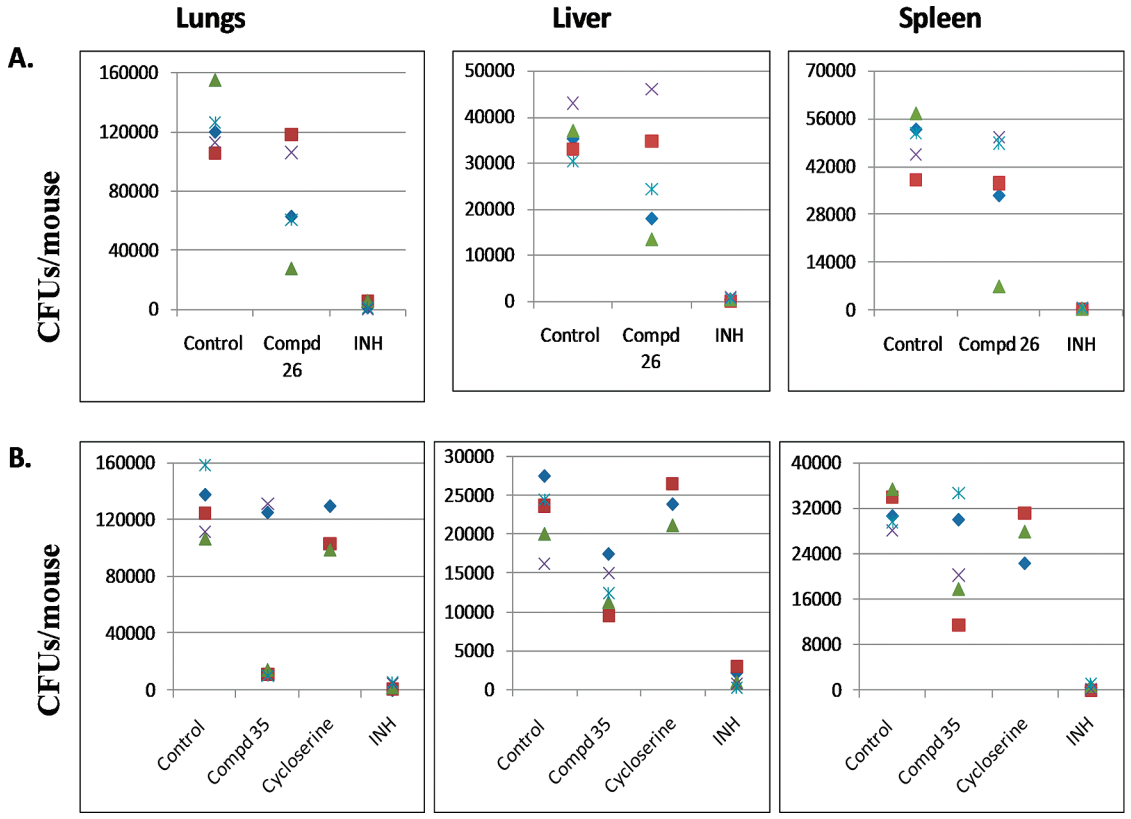

Figure 1. Activities of compound **26**, compound **35**, cycloserine, and INH in murine model of tuberculosis. In vivo efficacy of compound **26** (A), compound **35** (B), cycloserine, and isoniazid (INH) against *M. tuberculosis*. Mice (n = 5 or 3) were treated intraperitonially 5 days/week for five weeks. CFUs obtained from individual mouse organs (2 lungs, 1 liver, 1 spleen) are shown in the figure. Each symbol represents an individual mouse.

reduction of the CFU in the lungs) compared to the untreated control group. Compound **35** reduced the bacterial load to ~50% in four out of five mice in the liver tissues. In the spleens, compound **35** was slightly less effective, providing ~50% inhibition of the CFUs in only three mice. Reduction in the mycobacterial load in lungs and livers of mice treated with compound **35** was statistically significant ($P < 0.05$) compared to untreated mice. Mice administered compound **35** at 50 mg/kg for 5 weeks showed no adverse effects in terms of weight loss, behavioral changes, or in the findings of gross necroscopy after the mice were euthanized. It is notable that compound **35** showed moderate MIC values in vitro, it exhibited significant efficacy in vivo. Although compound **35** was less active than isoniazid both in vitro and in vivo, it exhibited similar in vitro and superior in vivo inhibition of mycobacterial replication in all organs (lungs, spleen, liver) as compared to another reference drug cycloserine (Figure 1B). It has been reported that 50−70% of cycloserine is excreted unchanged within 12−24 h following administration.[23] The improved in vivo inhibition exerted by **35** compared to cycloserine could be due to factors such as longer plasma half-life, higher stability, better bioavailability, better cellular delivery, and/or different mechanism of action.

The compounds **6**, **7**, **9**−**31**, **33**, **35** were also evaluated for their antibacterial activities against several Gram-positive and Gram-negative bacteria (*Staphylococcus aureus*, *Staphylococcus epidermis*, *Enterococcus fecalis*, *Bacillus subtilis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*). However, none of these compounds exhibited any antibacterial activity at concentrations up to 100 μg/mL except **18**, suggesting a preferential action against mycobacteria of the most active compounds (**24**−**26**, **33**, **35**). Compound 5-heptynyl-uracil (**18**) possessed activity only against the Gram-positive bacteria *S. epidermis* (MIC_{90} = 10 μg/mL), and *S. aureus*, *S. pyogenes*, *E. fecalis*, and *L. monocytogenes* (MIC_{90} = 50 μg/mL).

The precise mechanism of action of the active compounds inhibiting mycobacterial multiplication in this study is not clear. The complete genome sequence of *M. tuberculosis* has been deciphered.[24] It encodes many enzymes required for DNA and RNA synthesis and pyrimidine and purine nucleoside biosynthesis. It is postulated that active compounds may be inhibiting mycobacterial DNA and/or RNA synthesis by acting as substrates and/or inhibitors of metabolic enzymes of DNA and/or RNA synthesis. It is also possible that the active pyrimidinediones and pyrimidine nucleosides could be acting at two different mycobacterial targets and/or processes by virtue of dissimilarity in their structural features.

The promising compounds **23**−**27**, **33**, and **35** were tested in vitro for their toxicity for Huh-7 cells in concentrations up to 200 μg/mL using XTT and ^{3}H thymidine incorporation assays. No toxicity was observed except for compounds **27** (50% inhibition at 10 μg/mL concentration) and **33** (25% inhibition at 200 μg/mL by XTT assay). These studies confirm that the anti-TB activity of **23**−**26**, **33**, and **35** does not arise due to cytotoxicity of these compounds.

Conclusion

Tuberculosis is a significant global health problem and a high priority for research. There is an immense need to

discover novel agents for the treatment of tuberculosis, which work on targets and by mechanisms different from current therapies. A group of 5-alkyl, alkylalkynyl, and arylalkynyl analogues of pyrimidine and 2′-deoxy pyrimidine nucleosides were investigated for their antimycobacterial action. Compounds **23**−**26**, **33**, and **35** showed significant antimycobacterial activity in vitro. Compound **26** and **35** demonstrated encouraging in vivo efficacy and did not show in vitro or in vivo toxicity. These results suggest that there is an excellent potential in this class of compounds, particularly 5-arylalkynyl derivatives, to develop new antimycobacterial agents. Combination chemotherapy has been the most successful strategy for the treatment of tuberculosis. Therefore, new classes of compounds discovered in these studies have great potential to be used in combination with current TB drugs to shorten the duration of treatment, provide better compliance, reduce toxicity, and avoid or delay the emergence of resistance because they possess entirely different molecular structures and are expected to have different mechanisms of action.

Experimental Section

Melting points were determined with a Buchi capillary apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were determined for samples in Me_2SO-d_6, $CDCl_3$, or CD_3OD on a Bruker AM 300 spectrometer using Me_4Si as an internal standard. ^{13}C NMR (*J* modulated spin echo) spectra were determined for selected compounds where methyl and methyne carbon resonances appear as positive peaks and where methylene and quaternary carbon resonances appear as negative peaks. Chemical shifts are given in ppm relative to TMS as an internal standard. The assignment of all exchangeable protons (OH, NH) was confirmed by the addition of D_2O. All of the final compounds had >95% purity, determined by microanalysis. Microanalysis results 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 Machery-Nagel Alugram SiL G/UV silica gel slides (20 μM thickness). 5-Iodoracil (**5**), 5-iodo-2′-deoxyuridine (**32**), and 5-iodo-2′-deoxycytidine (**34**) were purchased from Aldrich.

Preparation of 5-Alkynyl-uracils. A full procedure is provided for 5-pentynyl-uracil (**17**). For other analogues, only brief spectroscopic data are presented.

5-Pentynyl-uracil (17). To a stirred solution of 5-iodoracil (**5**, 300 mg, 1.26 mmol) in anhydrous dimethylformamide (20 mL) at room temperature in a nitrogen atmosphere were added tetrakis(triphenylphosphine)palladium (0) (146 mg, 0.13 mmol), copper(I) iodide (48 mg, 0.25 mmol), diisopropylethylamine (0.44 mL, 2.52 mmol), and 1-pentyne (0.37 mL, 3.78 mmol). The reaction mixture was stirred at room temperature in a nitrogen atmosphere; the progress of the reaction was monitored by TLC in MeOH/$CHCl_3$ (10:90, v/v). After 18 h, 15 drops of 5% of the disodium salt of EDTA/H_2O were added to the reaction mixture, and the mixture was concentrated in vacuo. The residue obtained was purified on a silica gel column using MeOH/$CHCl_3$ (6:94, v/v) as an eluent to give **17** (200 mg, 89%) as a syrup. ^{1}H NMR (DMSO-d_6) δ 0.98 (t, *J* = 7.33 Hz, 3H, CH_3), 1.43−1.55 (m, 2H, β-CH_2), 2.32 (t, *J* = 7.02 Hz, 2H, α-CH_2), 7.64 (s, 1H, H-6), 11.16 (br s, 1H, NH), 11.28 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ 13.31 (CH_3), 20.73, 21.64 (2 × CH_2), 73.04 (C-β), 92.49 (C-α), 97.55 (C-5), 144.38 (C-6), 150.33 (C-2), 162.73 (C-4). Anal. ($C_9H_{10}N_2O_2$) C, H, N.

Compounds **18**−**26** were prepared using the procedure as described for **17**.

5-Heptynyl-uracil (18). This was obtained as a syrup in 86% yield. ^{1}H NMR (DMSO-d_6) δ 0.87 (t, *J* = 7.02 Hz, 3H, CH_3), 1.28−1.53 (m, 6H, 3 × CH_2), 2.33 (t, *J* = 7.02 Hz, 2H, α-CH_2), 7.64 (s, 1H, H-6), 11.15 (br s, 1H, NH), 11.28 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ 13.85 (CH_3), 18.75, 21.64, 27.89, 30.44 (4 × CH_2), 72.89 (C-β), 92.68 (C-α), 97.56 (C-5), 144.38 (C-6), 150.34 (C-2), 162.74 (C-4). Anal. ($C_{11}H_{14}N_2O_2$) C, H, N.

5-Decynyl-uracil (19). This was obtained as a solid in 89% yield; mp 228−230 °C (dec). ^{1}H NMR (DMSO-d_6) δ 0.85 (t, *J* = 6.72 Hz, 3H, CH_3), 1.20−1.50 (m, 12H, 6 × CH_2), 2.33 (t, *J* = 6.72 Hz, 2H, α-CH_2), 7.63 (s, 1H, H-6), 11.12 (s, 1H, NH), 11.28 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ 13.92 (CH_3), 18.77, 22.04, 28.18, 28.23, 28.47, 28.56 (6 × CH_2), 31.20 (α-CH_2), 72.88 (C-β), 92.67 (C-α), 97.56 (C-5), 144.33 (C-6), 150.33 (C-2), 162.72 (C-4). Anal. ($C_{14}H_{20}N_2O_2$) C, H, N.

5-Dodecynyl-uracil (20). This was obtained as a solid in 80% yield; mp 201−203 °C (dec). ^{1}H NMR (DMSO-d_6) δ 0.85 (t, *J* = 7.20 Hz, 3H, CH_3), 1.24−1.50 (m, 16H, 8 × CH_2), 2.33 (t, *J* = 7.20 Hz, 2H, α-CH_2), 7.62 (s, 1H, H-6), 11.20 (br s, 2H, 2 × NH). ^{13}C NMR (DMSO-d_6) δ 13.92 (CH_3), 18.78, 22.06, 28.19, 28.33, 28.49, 28.65, 28.73, 28.91 (8 × CH_2), 31.25 (α-CH_2), 72.91 (C-β), 92.63 (C-α), 97.53 (C-5), 144.46 (C-6), 150.38 (C-2), 162.72 (C-4). Anal. ($C_{16}H_{24}N_2O_2$) C, H, N.

5-Tridecynyl-uracil (21). This was obtained as a solid in 51% yield; mp 222−224 °C. ^{1}H NMR (DMSO-d_6) δ 0.85 (t, *J* = 7.02 Hz, 3H, CH_3), 1.24−1.49 (m, 18H, 9 × CH_2), 2.33 (t, *J* = 7.02 Hz, 2H, α-CH_2), 7.63 (s, 1H, H-6), 11.13 (br s, 1H, NH), 11.27 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ 13.93 (CH_3), 18.78, 22.07, 28.20, 28.23, 28.35, 28.52, 28.69, 28.93, 28.98 (9 × CH_2), 31.27 (α-CH_2), 72.89 (C-β), 92.67 (C-α), 97.58 (C-5), 144.36 (C-6), 150.34 (C-2), 162.73 (C-4). Anal. ($C_{17}H_{26}N_2O_2$) C, H, N.

5-Tetradecynyl-uracil (22). This was obtained as a solid in 72% yield; mp 232−234 °C (dec). ^{1}H NMR (DMSO-d_6) δ 0.85 (t, *J* = 7.02 Hz, 3H, CH_3), 1.23−1.51 (m, 20H, 10 × CH_2), 2.33 (t, *J* = 7.02 Hz, 2H, α-CH_2), 7.63 (s, 1H, H-6), 11.12 (s, 1H, NH), 11.28 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ 13.89 (CH_3), 18.75, 22.03, 28.17, 28.34, 28.47, 28.52, 28.65, 28.75, 28.88, 28.94 (10 × CH_2), 31.23 (α-CH_2), 72.87 (C-β), 92.64 (C-α), 97.58 (C-5), 144.28 (C-6), 150.29 (C-2), 162.67 (C-4). Anal. ($C_{18}H_{28}N_2O_2$) C, H, N.

5-(2-Phenylethynyl)-uracil[19] (23). This was obtained as a solid in 45% yield; mp >250 °C (dec). ^{1}H NMR (DMSO-d_6) δ 7.38−7.47 (m, 5H, aromatic), 7.90 (d, *J* = 5.8 Hz, 1H, H-6), 11.37 (d, *J* = 6.1 Hz, 1H, NH), 11.45 (s, 1H, NH). Anal. ($C_{12}H_8N_2O_2$) C, H, N.

5-(4-*n*-Propylphenylethynyl)-uracil (24). This was obtained as a solid in 42% yield; mp >250 °C (dec). ^{1}H NMR (DMSO-d_6) δ 0.88 (t, *J* = 7.33 Hz, 3H, CH_3), 1.52−1.64 (m, 2H, CH_2), 2.56 (t, *J* = 7.33 Hz, 2H, CH_2), 7.22 (d, *J* = 8.24 Hz, 2H, aromatic), 7.36 (d, *J* = 8.24 Hz, 2H, aromatic), 7.87 (s, 1H, H-6), 11.35 (s, 1H, NH), 11.42 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ 13.45 (CH_3), 23.63 (CH_2), 36.95 (CH_2), 81.87 (C-β), 91.56 (C-α), 96.94 (C-5), 119.75, 128.50, 130.82, 142.58 (C-phenyl), 145.29 (C-6), 150.22 (C-2), 162.28 (C-4). Anal. ($C_{15}H_{14}N_2O_2$) C, H, N.

5-(4-*n*-Pentylphenylethynyl)-uracil (25). This was obtained as a solid in 60% yield; mp >250 °C. ^{1}H NMR (DMSO-d_6) δ 0.85 (t, *J* = 7.02 Hz, 3H, CH_3), 1.23−1.33 (m, 4H, 2 × CH_2), 1.51−1.61 (m, 2H, CH_2), 2.58 (t, *J* = 7.63 Hz, 2H, CH_2), 7.21 (d, *J* = 7.94 Hz, 2H, aromatic), 7.35 (d, *J* = 7.94 Hz, 2H, aromatic), 7.87 (s, 1H, H-6), 11.35 (br s, 1H, NH), 11.41 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ 13.77 (CH_3), 21.79, 30.17, 30.72, 34.84 (4 × CH_2), 81.85 (C-β), 91.56 (C-α), 96.92 (C-5), 119.71, 128.44, 130.83, 142.83 (C-phenyl), 145.29 (C-6), 150.22 (C-2), 162.27 (C-4). Anal. ($C_{17}H_{18}N_2O_2$) C, H, N.

5-(2-Pyridylethynyl)-uracil (26). This was obtained as a solid in 87% yield; mp >250 °C. ^{1}H NMR (DMSO-d_6) δ 7.36 (m, 1H, aromatic), 7.51 (d, *J* = 7.93 Hz, 1H, aromatic), 7.81 (m, 1H, aromatic), 7.99 (s, 1H, H-6), 8.57 (d, J = 4.27 Hz, 1H, aromatic), 11.48 (br s, 2H, 2 × NH). Anal. ($C_{11}H_7N_3O_2$) C, H, N.

1,4-Bis-(2-pyridyl)-buta-1,3-diyne (27). This was obtained as a solid in 30% yield; mp 118−120 °C (dec). ^{1}H NMR (DMSO-d_6) δ 7.49−7.53 (m, 2H, aromatic), 7.75−7.79 (m, 2H, aromatic), 7.85−7.93 (m, 2H, aromatic), 8.65 (d, *J* = 4.88 Hz, 2H,

aromatic). ^{13}C NMR (CD_3OD) δ 73.61 (C-β), 81.57 (C-α), 125.83, 129.94, 138.53, 142.11, 151.13 (C-phenyl). Anal. ($C_{14}H_8N_2$) C, H, N.

6-Propyl-2,3-dihydrofuro-[2,3-*d*]pyrimidin-2-one (28). To a stirred solution of **17** (160 mg, 0.89 mmol) in methanol/triethylamine (7:3) (30 mL), was added copper(I) iodide (35 mg, 0.18 mmol) at room temperature in a nitrogen atmosphere. The reaction mixture was then heated to reflux and stirred for 3 h. The solvent was removed in vacuo. The solid thus obtained was redissolved in MeOH and filtered to remove the inorganic impurities. The filtrate was concentrated and purified on a silica gel column using MeOH/$CHCl_3$ (10:90, v/v) as an eluent to give **28** (80 mg, 50%) as a solid; mp >250 °C (dec). ^{1}H NMR (DMSO-d_6) δ 0.93 (t, J = 7.32 Hz, 3H, CH_3), 1.59−1.70 (m, 2H, β-CH_2), 2.60 (t, J = 7.32 Hz, 2H, α-CH_2), 6.37 (s, 1H, H-5), 8.14 (s, 1H, H-4), 11.98 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ 13.32 (CH_3), 19.77, 29.25 (2 × CH_2), 99.57 (C-5), 105.87 (C-4a), 138.74 (C-4), 155.68 (C-2), 157.42 (C-6), 171.93 (C-7a). Anal. ($C_9H_{10}N_2O_2$) C, H, N.

Compounds **29**−**31** were prepared using the procedure as described for **28**.

6-Pentyl-2,3-dihydrofuro-[2,3-*d*]pyrimidin-2-one (29). This was obtained as a solid in 64% yield; mp >250 °C (dec). ^{1}H NMR (DMSO-d_6) δ 0.87 (s, 3H, CH_3), 1.30 (br s, 4H, 2 × CH_2), 1.56−1.60 (m, 2H, β-CH_2), 2.59−2.62 (m, 2H, α-CH_2), 6.36 (s, 1H, H-5), 8.14 (s, 1H, H-4), 11.97 (br s, 1H, NH). Anal. ($C_{11}H_{14}N_2O_2$) C, H, N.

6-Octyl-2,3-dihydrofuro-[2,3-*d*]pyrimidin-2-one (30). This was obtained as a solid in 44% yield; mp 248−250 °C (dec). ^{1}H NMR (DMSO-d_6) δ 0.83−0.85 (m, 3H, CH_3), 1.24−1.35 (m, 10H, 5 × CH_2), 1.58−1.65 (m, 2H, β-CH_2), 2.60−2.64 (m, 2H, α-CH_2), 6.36 (s, 1H, H-5), 8.14 (s, 1H, H-4). Anal. ($C_{14}H_{20}N_2O_2$) C, H, N.

6-Decyl-2,3-dihydrofuro-[2,3-*d*]pyrimidin-2-one (31). This was obtained as a solid in 38% yield; mp >250 °C (dec). ^{1}H NMR ($CDCl_3$ + CD_3OD) δ: 0.83 (t, J = 7.02 Hz, 3H, CH_3), 1.18−1.36 (m, 14H, 7xCH_2), 1.62−1.67 (m, 2H, β-CH_2), 2.62 (t, J = 7.33 Hz, 2H, α-CH_2), 6.18 (s, 1H, H-5), 7.84 (s, 1H, H-4). Anal. ($C_{16}H_{24}N_2O_2$) C, H, N.

Compounds **33**, **35** were prepared using the procedure as described for **17**.

5-(2-Pyridylethynyl)-2′-deoxyuridine (33). This was obtained as a solid in 97% yield; mp 193−195 °C (dec). ^{1}H NMR (DMSO-d_6) δ 2.09−2.22 (m, 2H, H-2′), 3.62 (m, 2H, H-5′), 3.81 (m, 1H, H-4′), 4.25 (m, 1H, H-3′), 5.18 (t, J = 4.88 Hz, 1H, 5′-OH), 5.27 (d, J = 4.27 Hz, 1H, 3′-OH), 6.12 (t, J = 6.71 Hz, 1H, H-1′), 7.36 − 7.42 (m, 1H, aromatic), 7.53 (d, J = 7.93 Hz, 1H, aromatic), 7.83 (m, 1H, aromatic), 8.44 (s, 1H, H-6), 8.57 (d, J = 4.88 Hz, 1H, aromatic), 11.75 (s, 1H, NH). Anal. ($C_{16}H_{15}N_3O_5$) C, H, N.

5-(2-Pyridylethynyl)-2′-deoxycytidine (35). This was obtained as a solid in 81% yield; mp 224−226 °C (dec). ^{1}H NMR (DMSO-d_6) δ 1.98−2.22 (m, 2H, H-2′), 3.61 (m, 2H, H-5′), 3.82 (m, 1H, H-4′), 4.22 (m, 1H, H-3′), 5.13 (t, J = 4.88 Hz, 1H, 5′−OH), 5.22 (d, J = 4.27 Hz, 1H, 3′-OH), 6.13 (t, J = 6.71 Hz, 1H, H-1′), 7.08 (br s, 1H, N$\underline{H}_2$), 7.37 (m, 1H, aromatic), 7.77−7.87 (m, 3H, aromatic and N$\underline{H}_2$), 8.38 (s, 1H, H-6), 8.56 (d, J = 4.88 Hz, 1H, aromatic). Anal. ($C_{16}H_{16}N_4O_4$) C, H, N.

In Vitro Antimycobacterial Activity Assay (*M. bovis*, *M. tuberculosis*). *M. bovis* (BCG) and *M. tuberculosis* (H37Ra) were obtained from the American Type Culture Collection, Rockville, MD. The antimycobacterial activity was determined using the microplate Alamar Blue assay (MABA).[25] Test compounds were dissolved in DMSO at 100× 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, 5, 2.5, 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 medium 7H9GC and added to each well at 2.5 × 10^5 CFU/mL final concentration. Sixteen control wells consisted of eight with bacteria alone (B) and eight 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 spectrophotometer (at excitation 530/525 and emission 590/535) to determine OD values. If the B well became pink by 24 h (indicating growth), 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 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. Similar methodology was used for *M. bovis* BCG and *M. tuberculosis*. Rifampicin was used as a positive control. As negative controls, DMSO was added to the B well at a concentration similar to that in test compound wells; M wells served as negative controls. In most of the experiments, the M wells gave an OD of 3000−4000, and the B wells had OD values of 60000−100000.

In Vitro Antibacterial Activity Assay. Different bacterial strains were used for the determination of the in vitro antibacterial activity of the studied compounds. The in vitro antibacterial activity was studied by determining their minimum inhibitory concentrations (MICs) by means of the broth microdilution method. Briefly, exponentially growing bacteria were diluted in a liquid sterile medium to obtain a final inoculum of 1 × 10^4 CFU/mL and subsequently cultured with varying dilutions of compounds for 16−20 h. The MICs were defined as the lowest concentration at which bacterial growth was no longer evident.

In Vitro Cytotoxicity Assay. Human hepatoma cell line (Huh-7) was used to determine the effect of compounds **23**−**27**, **33**, and **35** on human cell cytotoxicity using XTT and ^{3}H-thymidine assays. Cell viability was measured using the cell proliferation kit II (XTT; Roche), as per manufacturer's instructions. Briefly, a 96 well plate was seeded with Huh-7 cells at a density of 1 × 10^5 cells per well. Cells were allowed to attach for 6−8 h when the medium was replaced with medium containing compounds at concentrations of 200, 100, 50, 10, and 1 μg/mL. DMSO was also included as control. Plates were incubated for 2 days at 37 °C. The color reaction involved adding 50 μL XTT reagents per well and incubating for 4 h at 37 °C. Plates were read on an ELISA plate reader (Abs 450−500 nm). For the ^{3}H-Tdr incorporation assay, Huh-7 cells were plated at 1 × 10^4 cells/well in 96-well flat bottom plates. Medium containing compounds at concentrations of 200, 100, 50, 10, and 1 μg/mL was added to the plate in triplicates. DMSO was also included as control. Plates were incubated for 2 days at 37 °C. The wells were pulsed with 0.5 μCi/well [^{3}H]-thymidine (Amersham) for 12−18 h. After this, the plates were harvested on filter papers (Perkin-Elmer) using a 96-well plate harvester (Tomtech MACH III M). The levels of [^{3}H]-thymidine incorporated into the DNA of proliferating cells were counted in a Microbeta Trilux liquid scintillation counter (Perkin-Elmer).

Animals and Infection. All animal experimental protocols used in this study were approved by the University of Alberta Animal Care and Use Committee for Health Sciences, and conducted in accordance with the guidelines of the University of Alberta, Edmonton, Canada, and the Canadian Council on Animal Care. Five−six week old female BALB/c mice were purchased from Charles River Laboratories and were allowed to acclimate for 1 week. Mice were inoculated iv in tail vein with 0.1 mL of bacterial suspension containing approximately 2.5 × 10^5 CFU of *M. tuberculosis* H37Ra in PBS. The infected mice were divided into groups (5 animals per group).

Administration of Drugs. The 5-(2-pyridylethynyl)-uracil (**26**) was dissolved in poly(ethylene glycol) methyl ether. The 5-(2-pyridylethynyl)-2′-deoxycytidine (**35**) and control drugs cycloserine and isoniazid were dissolved in 10% dimethyl sulfoxide and saline. The drugs were frozen at −20 °C and thawed each morning prior to administration. All of the compounds studied were administered intraperitonially. The compounds and dosages were: 5-(2-pyridylethynyl)-uracil (**26**), 50 mg/kg; 5-(2-pyridylethynyl)-2′-deoxycytidine (**35**), 50 mg/kg; cycloserine, 50 mg/kg; isoniazid, 25 mg/kg. Control animals received equivalent volumes of diluents only. Drug treatment was initiated 5 days after bacterial challenge and continued until week 6 (total treatment days = 25). Three days after the last treatment, mice were euthanized and lung, liver, and spleen were removed aseptically and individually homogenized. CFU counts per organ were determined on 7H11 selective agar plates purchased from BD Biosciences. The plates were incubated at 37 °C in ambient air for up to 4 weeks prior to counting the colonies.

Statistical Analyses. Standard randomization procedure was used in assigning the groups to mice after infection. To avoid bias, the identity of groups of animals was blinded from the person performing euthanization of mice and colony counts. Statistical analyses were done by independent sample *t* test using SPSS 16.0 software.

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