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Synthesis and Antimicrobial Evaluation of Deuterated Analogues of Metronidazole

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Abstract

Metronidazole is a commonly used antibiotic for anaerobic bacterial infections, protozoal infections, and microaerophilic bacterial infections by interacting with DNA and thereby inhibiting the protein synthesis. Metronidazole is administered orally, intravenously, or topically and has a half-life of 6.5 ± 2.9 hours. A number of studies have recently been conducted on the selective substitution of hydrogen with deuterium, which increases the bond strength thereby increasing the biological half-life and, consequently, the drug's metabolic stability. In an attempt to check whether the deuterated metronidazole possessed similar pharmacological activity as that of metronidazole, deuterated metronidazole was synthesised using deuterium oxide in the presence of benzoic acid under nitrogen atmosphere. The synthesised deuterated metronidazole was characterised by IR, ^{1}HNMR and mass spectroscopy and were tested for antibacterial, antifungal, and anti-tubercular activities. The metronidazole and its deuterated compound showed equipotent antifungal activity and aerobic antibacterial activity. Also, when compared with the non-deuterated compound, deuterated metronidazole exhibited better anaerobic antibacterial and anti-tubercular activity.

Keywords: Deuterated Metronidazole, Antibacterial Activity, Antifungal Activity, Anti-TB activity.

Introduction

The development of newer molecules is a time-consuming and expensive since only one out of 1, 00, 000 molecules become a potent lead. The reason for the low success rate is due to metabolic instability, poor absorption, oral bioavailability, and toxicity of the compounds. To overcome these challenges, medicinal chemists all over the world are working on various strategies such as bioisosteres, hybridisation techniques, scaffold hopping etc. One such recent technique is deuteration of molecules, which has gained interest in developing novel chemical entities with enhanced desired physicochemical properties [(1-7)].

Deuterium is a naturally occurring non-radioactive hydrogen isotope that was reported in 1932. Hydrogen has a mass of 1.008 atomic mass units (AMU) and includes one electron and one proton, but deuterium additionally contains a neutron and has a mass of 2.014 AMU. Deuterium has a natural abundance of around 1 part in 6400, or 0.015 percent, allowing huge amounts of deuterium to be extracted as heavy water (D_2O) with extremely high isotopic purity. D_2O can then be used as a direct or indirect source of deuterium in a variety of chemical reagents and building blocks for deuterated drug manufacturing.

Deuterium and hydrogen atoms are freely interchangeable in synthetic processes and deuteration leads to the replacement of deuterium for hydrogen in a molecule, Although the carbon- D bond is known to be stronger than the carbon-H bond due to lower zero-point energy, the kinetic isotope effect may occur as a result of the change in bond strength. When one or more hydrogen atoms are replaced with deuterium, the ratio can be as high as 6- to 10-fold. This process might have an impact on interactions between deuterated molecules and drug-metabolizing enzyme systems. Selective deuterium substitution preserves the pharmacologic profile of physiologically active compounds while also positively modifying their metabolic fate in some situations. Deuterium substitution can theoretically increase the safety, effectiveness, and/or tolerance of a medicinal drug [(8-14)].

Deutetrabenazine was the first deuterated pharmaceutical molecule approved by the FDA for the treatment of chorea, "an involuntary movement disorder" linked to tardive dyskinesia and Huntington's disease (Figure 1) [(15-18)].

The primary reason for D exchange is to enhance their metabolism and absorption characteristics, resulting in differentiated pharmaceuticals with enhanced safety and/or efficacy. Deuteration of bioactive chemicals is gaining popularity, with numerous deuterium-labelled entities now in clinical studies. As a result, scientists are developing new synthetic technologies for deuterated compounds in order to accommodate the increase in demand. Many deuterated compounds will not exhibit a beneficial

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and substantial exchange as compared to the precursor; yet, exhibiting a spectacular final product. Despite various obstacles, researchers are continuing to look at the possibility of replacing C-H with C-D to increase the active ingredient's half-life while simultaneously improving pharmacokinetics and toxicological characteristics. Recent papers focused on the deuteration-based synthesis of a new structural scaffold by converting the CH_3 group of metronidazole to the CD_3 group [19-22].

Metronidazole [2-(2-methyl-5-nitroimidazol-1-yl) ethanol] is a synthetic antibiotic derived from azomycin that was first discovered in Streptomyces spp. cultures in the 1950s. Actinobacteria, such as *Streptomyces eurocidicus* and *Nocardia mesenterica*, as well as Proteobacteria (*Pseudomonas fluorescens*), produce azomycin. Metronidazole is metabolised in the human liver to produce more polar metabolites. The gut microbiota can also modify metronidazole, resulting in reduced metabolites including hydroxyethyl oxamic acid and acetamide. Further literatures indicates that one of the metabolites like acetamide is the responsible for the cytotoxicity. Hence if the metronidazole is deuterated thereby the half-life will be increased and could result in reduction of cytotoxicity. The metabolism of metronidazole is explained in Figure 2 [23-27].

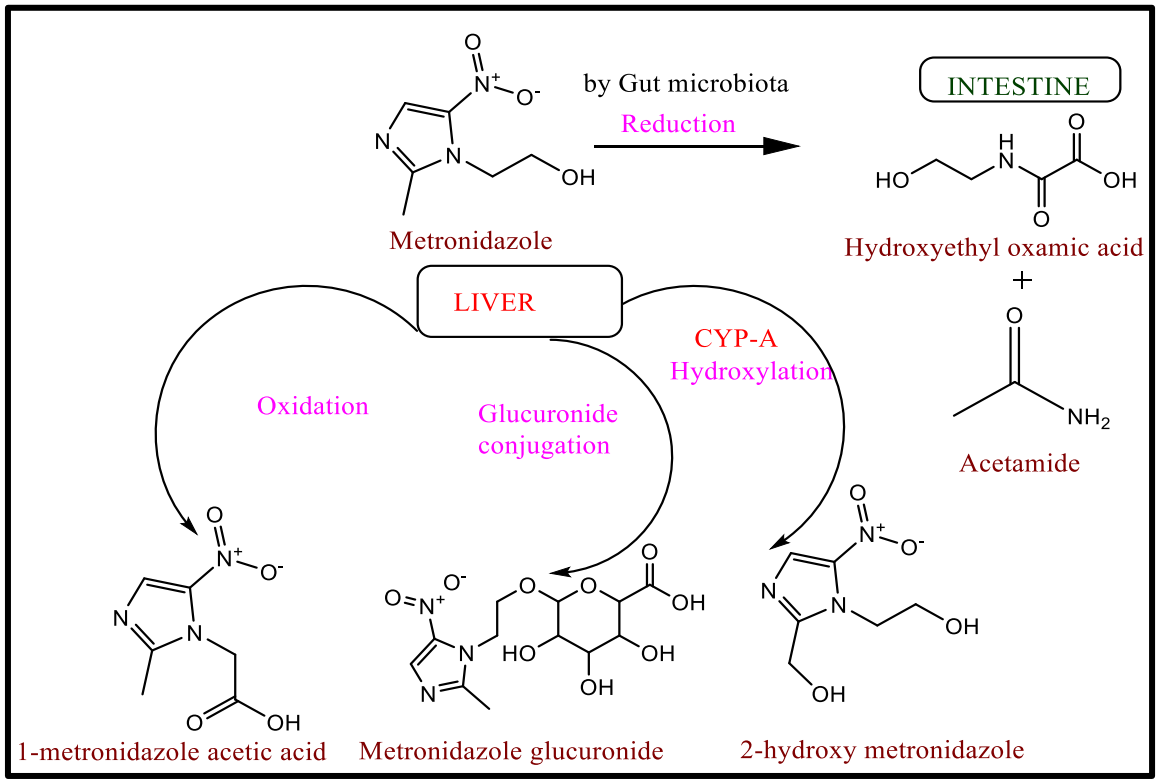


Figure 2. Metronidazole metabolism in humans

Metronidazole is now used to treat bacteria, including *clostridia* infections, *fusobacteria*, and *rosacea*, as well as oral and dental infections, septicemia, endocarditis, joint and bone infections, gynaecological infections, and respiratory tract infections. Even though it is widely used, metronidazole has been associated with neurotoxicity, genotoxicity, painful urination, cystitis, and pelvic pain due to long-term therapy (Figure 2). Tamoxifen, which is used to treat breast cancer causes genotoxicity but its deuterated derivative is found to be less genotoxic. Similarly, genotoxicity caused by metronidazole can be overcome by its deuterated analogue. Also, the half-life of metronidazole which is found to be 6.5 ± 2.9 hours can be increased in deuterated analogue due to increase in bond strength. In continuation to our ongoing work on the development of antimicrobial agents, we herein for the first time hitherto unreported deuterated analogues of metronidazole. The aim of the study is to convert the CH_3 group of metronidazole to the CD_3 group by deuterium exchange and to determine the physicochemical properties with their evaluation for anti-microbial activity [28-31].

Methods and Materials

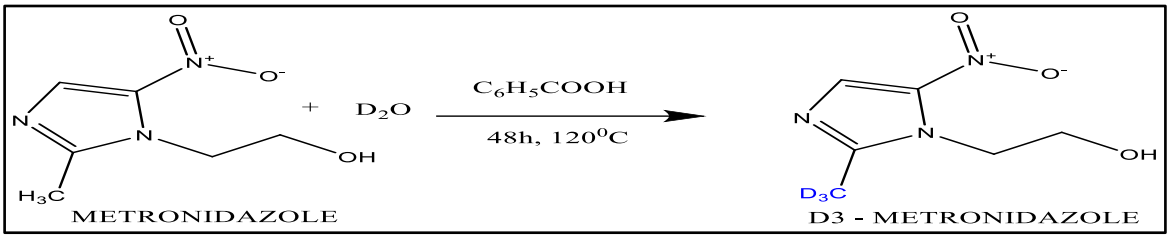
In silico studies

The physicochemical characteristics of metronidazole and deuterated metronidazole were investigated using Molinspiration (www.molinspiration.com) and Swiss ADME. The LogP parameter is used to determine whether or not the cell membrane is permeable. Drug absorption, bioavailability, drug-receptor interactions,

metabolism, and toxicity are all influenced by the hydrophilic or lipophilic properties of drug molecules. Total polar surface area (TPSA) is a good predictor of drug transport qualities such as intestinal absorption, bioavailability, and blood-brain barrier penetration because it is directly connected to a molecule's hydrogen bonding potential. A score of -1 to -4 on the Log S distribution is excellent for improved medication absorption and distribution in the body. The number of rotatable bonds is used to evaluate the molecular flexibility and the molecule with more rotatable bonds is more flexible, and its binding affinity with its binding pocket is better. Drug similarity data (Lipinski's rule of five) may be used to compare the molecule's characteristics and structural aspects to recognized medications. The bioactivity score for deuterated metronidazole and metronidazole like GPCR ligand, nuclear receptor ligand, a kinase inhibitor, and ion channel modulator is calculated using the Molinspiration drug-likeness score (www.molinspiration.com) [32- 35].

Synthesis of deuterated metronidazole

In a nitrogen environment, metronidazole (0.0342g 0.2 mmol), benzoic acid (0.0049g, 0.04 mmol), and 1mL of D_2O were refluxed for 48 hours at 120 ° C. The progress of the reaction was monitored by TLC. On completion, the mixture was neutralised with a saturated $NaHCO_3$ solution and extracted three times with ethyl acetate (3mL). The separated organic layers were combined, and anhydrous sodium sulphate were added to dry the organic layer. The organic layer was then distilled under reduced pressure to obtain crude product which were purified using 99.9% ethanol [36].



Scheme 1. Synthesis of deuterated metronidazole

Biological evaluation

The biological tests were carried out at the Central Research Laboratory at Maratha Mandal in Belgaum.

Test microorganisms

The test microorganisms that are considered for the study are Gram-positive (*E. faecalis, Staph aureus,* and *Fusobacterium nucleatum*), Gram-negative (*E. coli, Pseudomonas, Porphyromonas gingivalis,* and *Prevotella intermedia*), and Mycobacterium tuberculosis strain H37Rv. *Candida albicans* and *A. Niger* were also used to evaluate the antifungal activity of the newly synthesized deuterated compound.

Antifungal and aerobic antibacterial activity

Aerobic antibacterial and antifungal activities were carried out according to the protocols described in the literature [37].

Anaerobic antibacterial activity

Anaerobic antibacterial activity was performed as per the procedures cited in the literature [38].

Antitubercular activity

Anti-tubercular activity was carried out according to the protocols outlined in the literature [39-41].

Results and Discussion

Pharmacokinetics profile of metronidazole and its deuterated analogue

An orally active drug, according to Lipinski's rule of five, should contain no more than 5 hydrogen bond donors (OH and NH groups), not more than 10 hydrogen bond acceptors (mostly N and O), molecular weight under 500 g/mol, and a partition coefficient log P of less than 5. Metronidazole and deuterated metronidazole results were found to be compatible with Lipinski's 'Rule of Five,' with a molecular weight of 171.16 and 174.13 respectively.

Both metronidazole and its deuterated compound showed no violations according to Lipinski's rule and the results are as stated in Table-1.

Bioactivity score for metronidazole and its deuterated analogue

Molinspiration also predicted the bioactivity scores for metronidazole and its deuterated compound for drug targets, which are provided in Table 1. A molecule with a bioactivity score of greater than 0.00 is most likely to have significant biological activity, whereas values between -0.50 and 0.00 are considered moderately active, and scores less than -0.50 are considered inactive. Both metronidazole and its deuterated molecule had equal bioactivity scores for GPCR ligand, Kinase inhibitor, Nuclear Receptor Ligand,

Ion channel modulator, enzyme inhibitor, and Protease inhibitor. According to the data, metronidazole and deuterated metronidazole have a modest interaction with an enzyme receptor inhibitor.

Table 1. *In silico* studies for metronidazole and its deuterated analogue

Pharmacokinetics parameters	**Metronidazole**	**Deuterated metronidazole**
LogP	-0.47	-0.47
TPSA	83.88	83.88
natoms	12	12
nON	4	4
nOHNH	1	1
N violations	0	0
nrotb	3	3
MW	171.16	174.13
Bioactivity score		
GPCR ligand	-1.09	-1.09
Ion channel modulator	-0.87	-0.87
Kinase receptor inhibitor	-0.59	-0.59
Nuclear receptor ligand	-1.74	-1.74
Protease receptor inhibitor	-1.68	-1.68
Enzyme receptor inhibitor	-0.32	-0.32

LogP – Partition coefficient, TPSA- topological polar surface area, MW- molecular weight, nrotb-Number of Rotatable Bonds, nON - No. of hydrogen bond acceptor, nOHNH – no. of hydrogen bond donor.

Synthesis of deuterated metronidazole

Deuterated metronidazole was synthesised from commercially available metronidazole as starting material using D_2O at nitrogen atmosphere in the presence of benzoic acid. The synthesised deuterated metronidazole was obtained as a light brown solid with 90% yield; mp:166-168^{0}C; and R_f value of 0.6 (Toluene: Chloroform: Methanol (3.0:2.0:0.6 v/v)).IR (KBr, V_{max}) cm^{-1}: 1523: asymm. (NO_2) str., 1368: symm. (NO_2) str., 2856: (CD_3) str., 3123: (CH_2) str. of alkyl group, 3420: (OH) str. of alcohol. ^{1}H NMR (500 MHz,DMSO-d_6) 8.05 (s, 1H, imidazole), 7.45 (s, 1H, OH), 4.73 (t, 2H, O-CH_2, 4.63 (t, 2H, N-CH_2). Deuterated metronidazole showed an m/z of 174.

Aerobic antibacterial activity

The mass differences associated with the replacement of hydrogen by deuterium in a molecule are likely to have a major influence on the physical and chemical properties of the molecule. The results for the aerobic antibacterial activity of deuterated metronidazole and metronidazole against two Gram-positive bacteria (*Staph aureus and E. faecalis*) and two Gram-negative bacteria (*Pseudomonas and E. coli*) are shown in Table 2. From the results, it is clear that deuterated metronidazole exhibited better aerobic antibacterial activity against *E. faecalis,* with a MIC of 25µg/ml when compared with metronidazole 50µg/ml. The MIC values for metronidazole and its deuterated analogue showed similar values of 25µg/ml against *Staph aureus,* and 50µg/ml against *E. coli*. Bu in the case of *Pseudomonas*, deuterated metronidazole had a MIC of 50µg/ml, compared with 25µg/ml of metronidazole**.**

Anaerobic antibacterial activity

The anaerobic antibacterial activity of deuterated metronidazole and metronidazole against two Gram-negative bacteria (*Porphyromonas gingivalis* and *Prevotella intermedia*) and one Gram-positive bacteria (*Fusobacterium nucleatum*) are represented in Table 2. Deuterated metronidazole had a MIC of 0.8µg/ml in thioglycolate broth for anaerobic antibacterial activity in *Fusobacterium nucleatum*, and 3.12µg/ml in *Porphyromonas gingivalis* compared to 1.6µg/ml and 6.25µg/ml of metronidazole respectively. Also, in *Prevotella intermedia, deuterated* metronidazole showed a better MIC of 3.12µg/ml, compared with 6.25µg/ml of metronidazole. From the results, it is clear that deuterated metronidazole exhibited stronger anaerobic antibacterial activity against Gram-positive bacteria compared to Gram-negative bacteria. Deuterated metronidazole has superior antibacterial activity against anaerobic bacteria. This is due to the absence of electron transport proteins in aerobic cells with negative redox potential. As a result, the drug is only effective against bacteria with anaerobic metabolisms, even if it is effective against some microaerophiles like *H. pylori*.

Antifungal activity

The MIC values for antifungal activity of deuterated metronidazole and metronidazole are shown in Table 2. When tested for antifungal activity in Brain Heart Infusion Broth, both deuterated and its parent compound exhibited MIC values of 25µg/ml against candida and 100µg/ml against *A. Niger*. In comparison to *A. Niger*, both the compounds showed stronger antifungal activity against *candida.*

Antitubercular activity

The compounds were screened against *M. tuberculosis H37Rv* using the Microplate Alamar Blue Assay (MABA). Table 2 shows that deuterated compound, with a MIC of 1.6µg/ml, has superior anti-TB action than metronidazole, which has a MIC of 3.12µg/ml. Deuterated metronidazole's increased activity could be due to a primary isotope effect at the target site, in which the rupture of C-D bonds is directly involved, or the deuterated molecule's increased stability could play a role in the bacteria's inability to metabolise deuterated compound as easily as the protio form. When the antitubercular activity of deuterated metronidazole was compared to that of the standard drugs isoniazid and ethambutol, this showed similar activity with a MIC of 1.6µg /ml. In addition, when compared to the standard medication pyrazinamide, which has a MIC of 3.125µg /ml, deuterated showed significantly greater antitubercular activity.

Table 2. Antimicrobial activity of metronidazole and deuterated metronidazole

S. No	Microorganism	Minimum Inhibitory concentration (µg/ml)			
		Deuterated metronidazole	Metronidazole	Standard drug	
	Aerobic antibacterial activity			**Ciprofloxacin**	
1	*Pseudomonas*	50	25	<4	
2	*E. coli*	50	50	2	
3	*E. faecalis*	25	50	2	
4	*Staph aureus*	25	25	2	
	Anaerobic antibacterial activity			**Moxifloxacin**	
1	*Fusobacterium nucleatum*	0.8	1.6	<0.125	
2	*Porphyromonas gingivalis*	1.6	3.12	<0.125	
3	*Prevotella intermedia*	3.12	6.25	<0.125	
	Antifungal activity			**Fluconazole**	
5	*Candida*	25	25	16	
6	*A. Niger*	100	100	8	
	Anti-tubercular activity			**Isoniazid**	**Pyrazinamide**
7	*M. tuberculosis H37Rv*	1.6	3.12	1.6	3.125

Conclusion

To combat drug resistance, enhance pharmacokinetic profile and reduce cytoxicity of metronidazole, deuterated metronidazole was developed. The physicochemical parameters of metronidazole and its deuterated derivative were assessed using molinspiration and Swiss ADME, and both exhibited identical physicochemical properties and bioactivity scores. The antibacterial activity (aerobic and anaerobic) of metronidazole and its deuterated derivative against Gram-positive, Gram-negative bacteria, and fungus, as well as the M. tuberculosis H37Rv bacterium, was investigated. Deuterated metronidazole had a minimum inhibitory concentration (MIC) of 0.8 µg/ml in *Fusobacterium nucleatum*, compared to 1.6µg/ml for metronidazole, and a MIC of 1.6µg/ml in *Porphyromonas gingivalis*, compared to 3.12µg/ml for metronidazole. In *Prevotella intermedia*, deuterated compound had a MIC of 3.12µg/ml, whereas metronidazole had a MIC of 6.25µg/ml. Deuterated metronidazole's higher activity might be owing to a primary isotope effect at the target site, in which C-D bond breaking is directly involved. Because C-D bonds are more stable than C-H bonds, the molecule's enhanced stability may affect the rate of deuterated metronidazole metabolism. Similarly, when it comes to anti-TB activity, the deuterated compound, which has a MIC of 1.6µg/ml, is superior to that of metronidazole, which has a MIC of 3.12µg/ml. Deuterated derivatives were also shown to have nearly equivalent activity to metronidazole against Gram-positive and Gram-negative aerobic bacterial strains, as well as fungus. According to the findings, deuterated metronidazole is a good starting point for rational antibacterial activity design. In addition, pharmacokinetic and pharmacodynamic

investigations must be conducted to demonstrate the deuterated compound's activity. Despite the study and development of various deuterated medications, their efficacy, safety, and a complete understanding of the deuterated drugs' specific processes remain unsolved and difficult. Apart from that, designing and developing an effective deuterated medication with adequate efficacy remains a difficult task. Further study is going on to assess the long-term drug stability and toxicity studies for deuterated analogue of metronidazole. Despite all of these obstacles, deuterated medicines provide us with yet another method to tackle antimicrobial resistance.

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