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Delamanid is not metabolized by *Salmonella* or human nitroreductases: A possible mechanism for the lack of mutagenicity

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ABSTRACT

Nitro-containing compounds such as nitrofuran and nitroimidazole are drugs used for the treatment of infectious diseases. However, many of these nitro-containing drugs are positive in the bacterial reverse mutation assay (Ames test). The recently approved anti-multidrug-resistant tuberculosis (MDR-TB) drug, delamanid (Deltyba™; OPC-67683), a derivative of 4-nitroimidazole, was negative for mutagenicity in the Ames assay. In *Salmonella typhimurium*, mutagenicity of nitro compounds has been closely associated with the ability of nitroreductase to metabolize (degradation)these compounds. To explore the lack of mutagenicity for delamanid, we examined the initial metabolic rate and mutagenic-specific activity of a series of nitro compounds in *S. typhimurium* TA100. The order of maximum mutagenic-activity was nitrofuran > 2-nitroimidazole > 5-nitroimidazole ≥ 4-nitroimidazole, which is very similar to the order of initial metabolic rate, i.e., the Pearson's correlation coefficient ($r = 0.85$) showed a correlation between metabolic rate and mutagenic-activity. No metabolism of delamanid was detected even after 60 h of treatment. In addition, delamanid was not reduced by two human nitroreductases. These facts may explain the absence of genotoxicity of delamanid in both *in vitro* and *in vivo* tests.

1. Introduction

Nitro-containing compounds nitrofuran and nitroimidazole are used for the treatment of a wide variety of infectious diseases. They are not only used as an anti-bacterial [e.g., Furazolidone (FZD; 3-{[(5-nitro-2-furyl)methylene]amino}-1,3-oxazolidin-2-one)], but also as anti-protozoal [e.g., nifurtimox ((*R,E*)-3-methyl-4-{[(5-nitrofuran-2-yl)methylene]amino}thiomorpholine 1,1-dioxide)] agents. Furthermore, some nitroimidazoles have activity against fungi {satranidazole; [1-(1-methyl-5-nitroimidazol-2-yl)-3-methylsulfonylimidazolidin-2-one]} and HIV {DAMNIs; 1-[2(Diarylmethoxy) ethyl]-2-methyl-5-nitroimidazoles} (Mital, 2009).

While nitrofuran and nitroimidazole drugs are therapeutically active, risk of mutagenicity is a concern for the development of these compounds. Many of these drugs are positive in the bacterial reverse mutation assay (Ames test). Nitrofuran drugs such as furazolidone (FZD), and nitrofurantoin (NIT) are Ames positive agents and are banned for use in food-producing animals in the US (USFDA, 2014). Not all nitroimidazoles are mutagenic. Delamanid (DLM) (Deltyba™; OPC-67683), a recently approved anti-multidrug-resistant tuberculosis (MDR-TB) drug is a derivative of 4-nitroimidazole was not mutagenic in the Ames assay (Matsumoto et al., 2006). Furthermore, DLM was not genotoxic in a series of *in vitro* and *in vivo* studies. The *in vitro* genotoxicity tests include a bacterial reverse mutation assay (AMES test) and mouse lymphoma L5178/tk assay. The *in vivo* test is rat bone marrow micronucleus test. And in mice and rats, repeated oral administration of delamanid for up to 104 weeks did not appear to be carcinogenic (CHMP, 2013). Therefore, the mutagenicity of nitro compounds exhibit

Abbreviations: Multidrug-resistant tuberculosis, (MDR-TB); Furazolidone, (FZD); Nitrofurantoin, (NIT); Metronidazole, (MTZ); Benznidazole, (BZN); 2-nitroimidazole, (2NIT); 4-nitroimidazole, (4NIT); 1-(2-Aminoethyl)-2-methyl-5-nitroimidazole dihydrochloride, (12A); N-Benzyl oxymethyl-4-nitroimidazole, (NBenz); Delamanid, (DLM); CGI-17341, (CGI); OPC-37464, (464); 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide, (AF-2); Phosphate buffered saline, (PBS); Internal standard, (IS); High-performance liquid chromatography, (HPLC); Dimethyl sulfoxide, (DMSO); Trifluoroacetic acid, (TFA).

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different mutagenic potential. One key determinant of mutagenicity of nitro compounds may be the ability of nitroreductases to act on the nitro group in these compounds. Nitroreductase is capable of catalyzing the reduction of nitrosubstituted compounds using reducing co-factors (Oliveira et al., 2010). In *Salmonella typhimurium*, mutagenicity of nitro compounds was closely associated with nitroreductases SnrA and Cnr (Salamanca-Pinzón et al., 2006; Watanabe et al., 1989; Yamada et al., 1997). The Cnr nitroreductase knockout strain of *S. typhimurium* showed lower sensitivity whereas Cnr high-expression strain of *S. typhimurium* showed higher sensitivity to nitro mutagenic compounds than TA100 (Watanabe et al., 1989; Yamada et al., 1997). SnrA is also a nitroreductase which is mainly responsible for the reduction of nitrofurans in *S. typhimurium* (Salamanca-Pinzón et al., 2006). The present study examined the initial metabolic rate by *S. typhimurium* and mutagen specific activity of four groups of nitro aromatic skeletons; two nitrofurans, two 2-nitroimidazoles, two 5-nitroimidazoles, and five 4-nitroimidazoles. We then examined whether DLM can be degraded by human nitroreductases. Our findings suggest that the metabolism of nitro compounds by *S. typhimurium* strongly correlates with mutagenic activity. The fact that DLM is not degraded by human nitroreductases may explain why it has no genotoxicity in both *in vitro* and *in vivo* testing.

2. Materials and methods

2.1. Compounds and enzymes

The nitro compounds we used in this study are listed in Table 1. FZD, NIT, metronidazole (MTZ), and benznidazole (BZN) were purchased from Sigma Aldrich (St Louis, MO, USA). 2-Nitroimidazole (2NIT), 4(5)-Nitroimidazole (4NIT), and 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2) were purchased from Wako Pure Chemical (Osaka, Japan). 1-(2Aminoethyl)-2-methyl-5-nitroimidazole dihydrochloride (12A) was purchased from Kanto Chemical (Kanto Chemical, Tokyo, Japan). N-Benzyl oxymethyl-4-nitroimidazole (NBenz) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). DLM, CGI-17341 (CGI), and OPC-37464 (464) were synthesized at Otsuka Pharmaceutical (Tokyo, Japan). Since 4-nitroimidazole and 5-nitroimidazole are tautomer, it could not be categorized in 4-nitroimidazole nor 5-nitroimidazole group. We made one group 4(5)-nitroimidazole for this molecule. Two human nitroreductases NQO1 and NQO2 (recombinant, expressed in *Escherichia coli*) were also purchased from Sigma Aldrich. The activities of NQO1 and NQO2 were 558 and 617 units/mg, respectively.

2.2. Ames assay

The Ames test was performed based on the description with the Organisation for Economic Co-operation and Development Test Guideline plate incorporation method (OECD, 1997). *S. typhimurium* TA100 from frozen stock were pre-cultured in OXOID Nutrient Broth No.2 (Thermo Scientific, Waltham, MA, USA) for 8 h at 37 °C. The culture was measured OD600 and confirmed the OD600 value was more than 2.5. The culture was centrifuged and the pellet was resuspended with the same amount of phosphate buffered saline (PBS). Test compounds were adjusted to 5 mg/mL with dimethyl sulfoxide (DMSO) and were diluted 4-fold with DMSO. The final doses used in the assay were 0.00119–5000 μg/plate. For positive control, 0.1 μg/mL (0.01 μg/plate) AF-2{2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide} was used and for negative control, DMSO was used (USFDA, 2000). A 2.0 mL volume of top agar (Bacto agar: 8.0 g/L, NaCl: 5.0 g/L, biotin: 0.05 mmol/L L-histidine: 0.05 mmol/L) was mixed with a 0.1 mL volume of each test and reference compound and then spread onto Tesmedia® AN (Oriental Yeast, Tokyo, Japan). Each test plate was duplicated. The plates were inverted and incubated at 37 °C for 48 h. Thenumbers of revertants on the plates were counted. Only plates without precipitation were used in this study. The result was classified as Ames positive only if the plate had twice the number of colonies of the negative control plate. In addition, to quantitatively compare the mutagenic activity, the mutagen specific activity of each Ames positive concentration was calculated. The calculation was conformed to the Japanese Industrial Safety and Health Act, Article 57 (MHLW, 1972). The values were calculated as dividing number of increased colonies by the amount of the test compound. Then, the highest value was selected (maximum mutagen specific activity).

Mutagen specific activity:

$$\frac{\textit{Average colonies on the test plate} - \textit{Average colonies on the negative control plate}}{\textit{Amount of the test compound}\left(\frac{nmol}{plate}\right)}$$

2.3. Metabolism of nitro compounds by S. typhimurium

S. typhimurium TA100 from frozen stock were pre-cultured in Oxoid nutrient broth No.2 (Thermo Scientific) for 16 h. The culture was centrifuged and the pellet was re-suspended with the same amount of PBS. The nitro compounds were dissolved in DMSO to prepare 1.0 mM stock solutions, which were then diluted at a 1:100 ratio with the bacterial culture or PBS (negative control). The mixtures were incubated in an incubator set at 37 °C for 1–3600 min, and then the compounds were extracted with twice the volume of an acetonitrile solution containing the internal standard (IS). The samples were centrifuged and the supernatants were stored at −15 °C or lower until measurement. The concentration of nitro compounds in the samples was measured by high-performance liquid chromatography (HPLC) analysis.

2.4. Reduction assay with human nitroreductases

The 3 μM DLM and FZD in PBS were each mixed with NQO1 or NQO2 in the presence of 0.5 mM βNADH and were incubated at 37 °C for 2–24 h. The samples were added to twice the amount of an acetonitrile solution containing an IS. To assess the spontaneous degradation of DLM and FZD in PBS, samples without NQO1 and NQO2 were also examined.

2.5. HPLC analysis

HPLC analysis was carried out with the SCL-10A VP HPLC system