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Research Article

Mutagencity, Cytotoxicity and DNA Damage Study in Mutant *Salmonella typhemurium* and HL 60 Cell Line Treated with a Potent (R)-3,5-Dinitro-N-(1-(p-tolyl)ethyl) Benzamide(DNB)

Amit Kumar[1,2], Rakesh Kumar Nagar[1,2], Gurunadham Munagala[1,3], Arem Qayum[1,4], Shashank K Singh[1,4], Parvinder Pal Singh[1,3], Narendra Singh Chauhan[1,2], Shah Nawaz[1,2], Arfan Khalid[1,2], Irfan Qasam[1,2] and Govind Yadav[1,2,5*]*

[1]*Academy of Scientific & Innovative Research (AcSIR), CSIR-Indian Institute of Integrative Medicine, India*

[2]*Mutagenicity Laboratory, CSIR-Indian Institute of Integrative Medicine, India*

[3]*Division of Medicinal Chemistry, CSIR-Indian Institute of Integrative Medicine, India*

[4]*Division of Cancer Pharmacology, CSIR-Indian Institute of Integrative Medicine, India*

[5]*Central Laboratory Animal Facility, CSIR-Indian Institute of Integrative Medicine, India*

Abstract

The mutagenic potential of newly synthesized (R)-3,5-Dinitro-N-(1-(p-tolyl)ethyl)Benzamide(DNB) was assayed by the Bacterial reverse Mutation assay (AMES) at the concentration of 0, 0.32, 1.6, 8, 40, 200, 1000 and 5000 µg/plate without (-S9) and with (+S9) metabolic activation (as per OECD471). Mutagenicity was assessed using histidine dependent (his-), base pair substitution specific *Salmonella typhimurium* strains carrying mutation in hisG gene of TA100 (hisG46), TA1535 (hisG46), TA102 (hisG428) and frameshift mutation specific strains carrying mutation in hisD and hisC gene of strain TA98 (hisD3052), TA1537 (hisC3076), respectively. DNB was also assessed for Mutagenicity using Tryptophan dependent *E. coli WP2uvrA* strain. The Mutagenic Index (MI) increased dose dependently in strains TA1535 (1.01-16.39, 1.01-14.58), TA100 (1.66-2.53, 1.55-2.46), TA102 (1.0-1.56, 0.95-2.04). Whereas no dose dependent increased in MI was observed in non-mutagenic strains TA98, TA1537 and base pair specific *E. coli WP2uvrA*, irrespective of S9 metabolic activation. Results of the mutagenicity study revealed that DNB causes mutagenicity only in base pair substitution specific *S. typhimurium* strain TA100, TA1535, TA102, carrying mutation in hisG gene of histidine operon. Findings observed in mutagenicity study also reflects in DNA damage study (COMET assay) of DNB as 39.19%, 34.79% DNA obtained in tail of the comet at 0.22 mM, 0.04 mM conc., respectively, indicate DNA damage as compared to untreated HL60 cells. This study provides useful information on mutagenic and genotoxic properties of the DNB that indicates possible carcinogenicity of the nitro group containing potent Anti TB compound evaluated in this study.

Keywords: (R)-3,5-dinitro-N-(1-(p-tolyl)ethyl)benzamide (DNB); Bacterial reverse mutation assay; Mutagenic and genotoxic properties

Introduction

Moving a drug candidate through the developmental pipeline from the discovery phase to clinical development and eventual market approval requires diverse expertise and effective coordination of preclinical activities, including mutagenicity and genotoxicity assessments. Mutagenic chemical/pharmaceuticals that can induce mutation can potentially damage the germ cell line leading to fertility problems and to mutations in future generations, mutagenic compounds are also capable to induce cancer [1-7] and this concern has driven most of the mutagenicity testing programmes [8-10]. The identification of chemicals capable of inducing mutation has become an important procedure in safety assessment in development of chemicals having potential as chemotherapeutic agents.

The Bacterial reverse mutation assay (Ames assay) measures the direct mutation potential of a test article using bacterial cells that carry point mutations in genes, required to synthesize the amino acid histidine and tryptophan, accordingly. The bacterial reverse-mutation assay with strains of *S. typhimurium* and *Escherichia coli* (Ames test) has been used extensively in mutagenicity testing (Base pair substitution and frameshift mutation) which have GC base pairs at the primary reversion site (*S. typhimurium* TA98, TA100, TA1535, TA1537) and which have an AT base pair at the primary reversion site (*E. coli WP2uvrA* strains or *S. typhimurium* TA102) in order to be sensitive to a broad range of chemicals without and with metabolic activation [8,11].

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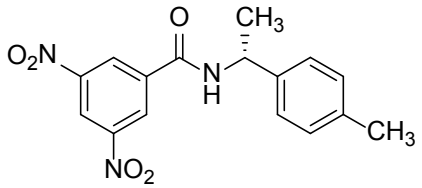
***Corresponding author:** Govind Yadav, Academy of Scientific & Innovative Research (AcSIR), CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu 180001, India, Tel: +91-191-2585006-13 Ext 336; Fax: +91-191-2586333; E-mail: gyadav@iiim.res.in

Parvinder Pal Singh, Division of Medicinal Chemistry, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu 180001, India, Tel: +91-191-2585006-13 Ext 336; Fax: +91-191-2586333; E-mail: ppsingh@iiim.res.in

Tuberculosis (TB) is one of the most ancient and infectious diseases of mankind and claims more human lives every year than any other bacterial infection. Tuberculosis is caused by Mycobacterium tuberculosis. It affects about 30% of world's population. The Emergence of Multidrug Resistant TB (MDRTB) or tuberculosis that are resistant to rifampicin and isoniazid has further complicated the situation to control tuberculosis. Every year, about 8 million people are infected and 2-3 million people die due to TB. Due to more claims of human lives it necessary to develop a novel anti-tuberculosis agent.

Designing of safer molecule for the treatment of tuberculosis is the priority of pharmaceutical industry. The success of 2 nitroimidazoles and 2-nitroimidazo-oxazine PA824 [15,16] are being developed by the TB Alliance and the dihydroimidazooxazole OPC67683 [17], which is being developed by Otsuka Pharmaceutical. Of note, this class of compounds can trace its history back when development of lead compound (CGI-17341) from Ciba-Geigy series of nitroimidazoles, was halted due to its mutagenic activity in AMES assay. It was not until the 1990's that the small pharmaceutical company Pathogenesis explored this series further, modifying the furan ring structure of the Ciba-Geigy compounds and thereby describing the nitroimidazo-oxazines, or nitroimidazopyrans. Pathogenesis studied 1700 novel compounds and determined that PA824 was the most active of these compounds against *M. tuberculosis* in a murine infection model [12]. PA824 was found not to be mutagenic in the AMES assay, both with and without S9 activation, but also by chromosomal aberration, mouse micronucleus, and mouse lymphoma tests, all of which have been negative [13]. Furthermore, PA824, OPC67683 shows no evidence of mutagenic potential [14]. Therefore, this study was planned to investigate mutagenic potential and structure activity of a newly synthesized potent anti-Tuberculosis compound (R)-3,5-dinitro-N-(1-(p-tolyl)ethyl)benzamide(DNB analog) showing potent anti-Tuberculosis activity.

Materials and Methods



Structure of DNB- (R)-3,5-dinitro-N-(1-(p-tolyl)ethyl)benzamide

Test compound: (R)-3,5-dinitro-N-(1-(p-tolyl)ethyl)benzamide was designed and synthesized in house in Medicinal chemistry division, Indian institute of integrative medicine. The compound under test is a synthetic analog of N-alkylphenyl-3,5-dinitrobenzamide and its Anti-tuberculosis activity against sensitive strain (H37Rv) and resistant strains of MTB was assayed by Clinical microbiology division, Indian institute of integrative medicine (data not shown) and published earlier by Gurunadham munagala et al. [15].

Chemicals: Dimethyl Sulfoxide (DMSO), Nicotinamide adenine dinucleotide phosphate sodium salt, D-Glucose-6-phosphate disodium salt, Magnesium chloride-Histidine monohydrate, D-Biotin, L-Tryptophan, Sodium azide, 2-Aminoanthracene, Methylmethane sulfonate, D(+) Glucose, Potassium phosphate dibasic anhydrous, Citric acid monohydrate, magnesium sulfate, Sodium ammonium phosphate, Monobasic sodium phosphate, Dibasic sodium phosphate, Sodium chloride were purchased from Sigma aldrich.co. Oxoid nutrient broth no. 2 (oxoid) and Bacto Agar (BD Bacto USA) were used in preparation of solutions and culture media. Aroclor 1254 induced Rat liver metabolic activation system procured from Moltox, pvt, ltd, USA. MTT dye was purchased from sigma Aldrich.co.

Bacterial Strains and mutation specificity

Bacterial strains (procured from Moltox pvt ltd) used in the Bacterial reverse mutation assay to screen compound for Base pair substitution were *S. typhimurium* TA100, TA1535, TA102, *E. coli WP2uvrA* and for Frame shift mutation *S. typhimurium* TA98, 1537. Mutational specificity of tester strains (Table 1) having GC base pair at primary reversion site *viz. S. typhimurium* TA100,TA1535,TA98,1537 and AT base pair at primary reversion site *S. typhimurium* TA102 and *E. coli WP2uvrA* was earlier specifically confirmed, specified and described [1,11,16-24]. All the tester strain were checked for their genetic integrity by phenotypic characterization as Histidine dependence to detect mutation in histidine operan, Biotin dependence to detect uvrB deletion that makes defective DNA repair mechanism in uvrB-bio genes, crystal violet sensitivity for rfa mutation of lipo-polysaccharide layer that makes bacteria more permeable to bulky chemical, ampicillin resistance for the presence of pKM101,tetracycline resistance for pAQ1 and found as recommended by OECD471 and described earlier by Mortelmans and Zeiger [11].

Metabolic activation system (S9 Mixture)

The S9 fraction (Post mitochondrial metabolic activation system) prepared from Sprague-Dawley/ Wistar rat treated with the polychlorinated biphenyl mixture Aroclor 1254 (500 mg/kg Body weight) was purchased from Molecular Toxicology Inc (Boone NC USA).

The metabolic activation system consisted of 5% of S9 fraction, 1% of 0.4 M $MgCl_2$, 1% of 1.65 M KCl, 0.5% of 1 M D-glucose-6-phosphate disodium salt, 4% of 0.1M NADP, 50% of 0.2 M Phosphate buffer and 39.5% of sterile distilled water.

Bacterial reverse mutation assay procedure

Mutagenic activity was tested by *Salmonella*/microsome assay by using the *S. typhimurium* tester strain (histidine auxotrophic) TA98, TA100, TA1535, TA1537 & TA102 and Escherichia coli (Tryptophan auxotrophic) tester strains *WP2uvrA*, kindly provided by Molecular Toxicology Inc. (Boone NC USA) with and without metabolic activation system by the plate incorporation method. The strain from frozen culture were grown overnight for 12-14 h in Oxoid Nutrient Broth No. 2. The metabolic activation system (S9) was prepared freshly before each test. Seven different doses of test compound were prepared in DMSO. The concentration of test compound was selected on the basis of a preliminary toxicity dose determination assay. The test compound was evaluated over a thousand fold range of concentration, starting from the main stock of 50 mg/ml to the decreasing level by a dilution factor of 5. All experiments were analyzed in triplicate with each test dose as well as negative and positive control. In the entire subsequent assay, the upper limit of the dose range tested was either the highest non-toxic dose or the lowest toxic dose determined in this preliminary assay. Toxicity may be considered as a reduction of no. of revertants (histidine/ tryptophan auxotrophs) compare to no. of revertants in negative control. The various concentration of test compound to be tested were added to 0.5 ml of 0.1 M phosphate buffer (pH 7.4) or with 0.5 ml of 5% S9 mixture (without & with S9 respectively) and 0.1 ml of overnight grown bacterial culture. The 2 ml of top agar was added according

to the tester strains and then poured onto a plate containing minimal glucose agar. The plates were incubated for 48 h and the revertants colonies were counted manually. The mutagenic index was also calculated for each concentration tested, this being the average no. of revertants per plate with the test compound divided by the average no. of revertants per plate with negative (solvent) control. A compound was calculated mutagenic when a dose-response relationship was detected and a two fold increase in the number of mutant (MI>2) was observed with at least one concentration. The standard mutagen used as positive control in experiments without S9 is 2. Nitrofluorene for TA98 (7.5 µg/plate), Sodium azide for TA100 & TA1535 (5 & 0.5 µg/plate respectively) and MMS for *E. coli WP2uvrA* (2.5 µl/plate) and 2-Aminoanthracene (2.5 µg/plate for TA98 & TA1535, 5µg/plate for TA100 and 10 µg/plate for *E. coli*) was used with S9. DMSO served as a negative control (100 µl/plate).

Comet assay

Comet assay measures, single as well as double strand breaks, alkali liable sites oxidative DNA base damage, DNA-DNA/DNA-Protein/DNA-Drug cross linkage and DNA repair. The principle of the assay rests on strand breakage of the super coiled double helical DNA which leads to the reduction in the size of large molecules where in strands of the DNA can be stretched out by electrophoresis. Similarly, under highly alkaline conditions there is denaturation, unwinding of the double strand and the expression of alkali labile sites as single strand breaks. Comets form when the broken ends of the negatively charged DNA molecule become free to migrate in the electric field towards the anode. DNA migration is a function of both the size and the number of broken ends of the DNA. Due to the damage, the tail increases in length and reaches its maximum which is dependent on the electrophoretic conditions and not on the size of the fragments [25]. In order to quantify the DNA damage induced by test compounds, comet assay was performed by the method given in the literature [26].

Exponentially growing HL-60 cells (2×10^6 cells/ml/well) were treated with different concentrations of test compounds and incubated for 24 h. The cells were harvested and washed with PBS and its 50 µL aliquot was embedded in 100 µl of warm (45°C) low melting point agarose (0.75%). The resulting mixture was spreaded over precoated microscopic slides (1% agarose). The gel was covered with glass cover slip and left to set at 4°C for 20 mins to 30 mins. Gel embedded cells were lyesd in lysing buffer (2.5M NaCl, 100 Mm disodium EDTA, 10 mM Trizma base, 8 g/l NaOH pH 10) for 2 hours at 4°C to allow DNA unwinding. Electrophoresis was performed at 300 mA and 24 Volts (~0.74 V/cm) for 30 min. The slides were stained with 100 µl 1X Ethidium bromide, leave for 10 min and then dipped in Chilled distilled water to remove excess stain and examined under an Olympus fluorescence microscope (1X41) equipped with an excitation filter (BP 510 nm) and a barrier filter (590 nm). Slides were analyzed using computerized imaging analysis system (KOMET 5.5). To evaluate the amount of DNA damage, computer generated tail movement values were used. Approximately hundred cells were used to access the DNA damage by (a) olive tail movement (b) tail length (c) tail coefficient variance.

Cell proliferation assay

The HepG2 cells (maintained in house lab) at 70% confluence were treated with (R)-3,5-dinitro-N-(1-(p-tolyl)ethyl) benzamide at various concentration ranging from 0.0256 µg to 10000 µg for 48 h. MTT dye (2.5 mg/ml) was added 4 h prior to the termination of experiment. MTT formazon crystals were dissolved in 150 µl of DMSO and absorbance was measured at 570 nm.

Results

Table 2 shows the results of the mutagenicity study as mean number of revertants/plate, standard deviation (M ± SD) of the colony counts in three plates of each concentration(0, 0.32, 1.6, 8, 40, 200, 1000 and 5000 µg/plate) of anti-TB compound (R)-3,5-dinitro-N-(1-(p-tolyl) ethyl)benzamide(DNB analog) without(-S9) and with(+S9) Aroclor induced Rat liver S9 (4%) metabolic activation in the bacterial reverse mutation assay using *S. typhimurium* TA98, TA1537, TA100, TA1535, TA102 and *E. coli WP2uvrA* strains each carrying different mutation in various genes.

Two or more times increases in the number of his+ revertants compared to negative control (solvent) was observed and therefore (R)-3,5-dinitro-N-(1-(p-tolyl)ethyl)benzamide(DNB analog) considered as mutagenic compound and concentration dependent increases in his+ revertants even less than two times was considered as the sign of mutagenicity. The standard mutagen used as positive control in experiments without S9 was 2-Nitrofluorene for TA98 (7.5 µg/plate), Sodium azide for TA100 & TA1535 (5 & 0.5 µg/plate respectively) and MMS for *E. coli WP2uvrA* (2.5 µl/plate) and 2-Aminoanthracene (2.5 µg/plate for TA98 & TA1535, 5 µg/plate for TA100 and 10 µg/plate for *E. coli*) was used with metabolic activation (+S9), shows high number of his+ revertant as depicted in Table 2.

Mutagenicity in base pair substitution mutation sensitive *Salmonella* and *E. coli* strains Results in the form of mutagenic index which were depicted in Table 3 were after treatment with test compound using base pair substitution mutation specific *S. typhimurium* strains (TA100, TA1535, TA102) carrying mutation at hisG gene (hisG46, hisG428), showed mutagenicity as concentration (doses of test compound) dependent increases in Mutagenic Index (MI) without and with metabolic activation. Other hand no concentration dependent increased in Mutagenic index was observed in case of *E. coli WP2uvrA*; rather MI was decreased from 1.11/1.0 to 0.67/0.62 at 0.32 µg/plate to 5000 µg/plate of test compound without and with metabolic activation, respectively.

hisG46 mutant site carrying mutation in strains TA100 and TA1535 reverted to wild type state after treatment with mutagen (R)-

Table 1: Type of mutations and characteristics of *Salmonella typhimurium/E. coli WP2uvrA* strains are summarized as follows.

***Salmonella typhimurium/E. coli* Strains**					
Mutation(hotspot)	**Strains**	**Characteristics/ Plasmid**	**Base pair at primary reversion site**	**DNA target/ Reversion event**	**Ref.**
hisG46	TA100	bio.rfa-.uvrB-.pKM101	G/C	-G-G-G Base pair substitution	21
hisG46	TA1535	bio.rfa-.uvrB	G/C	-G-G-G Base pair substitution	21
hisG428	TA102	rfa-.pKM101.pAQ1	A/T	TAA(Ochre) Base pair substitution	28
hisD3052	TA98	bio.rfa-.uvrB-.pKM101	G/C	-C-G-C-G-C-G-C-G frameshift	24
hisC3076	TA1537	rfa-.uvrB	G/C	+1 frameshift	1
TrpE56	E. coli Wp2uvrA	bio.pKM101uvrA	A/T	(near-C-C-C-run) Base pair substitution	

Table 2: Mutagenic activity expressed as the mean and standard deviation of the number of revertants (his+) in strains, specific for frameshift mutation(TA98, TA1537) and Base pair substitution(TA100, TA1535, TA102) *S. typhimurium* and *E. coli WP2uvrA* strains exposed to R)-3,5-dinitro-N-(1-(p-tolyl)ethyl) benzamide(DNB) at various doses, without(-S9) and with(+S9) metabolic activation.

Name	Conc µg/ plate	*S. typhimurium and E coli* Mutated strains having GC/AT Base pair at Primary reversion site											
		GC base pair at primary reversion site								AT Base pair at reversion site			
		Frame shift mutation specific				Base pair substitution specific				Base pair substitution			
		TA-98 hisD3052		TA1537 hisC3076		TA100 hisG46		TA1535 hisG46		TA102 HisG428		*E. coli WP2uvrA*	
		-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Neg.	0	44 ± 1.15	58 ± 4.04	21 ± 3.05	25 ± 2.08	221 ± 5.77	241 ± 7.02	21 ± 0.57	24 ± 1.15	268 ± 5.5	293 ± 6.11	40 ± 3.21	45 ± 2.08
Pos. Control		1185 ± 5.03	1298 ± 9.16	1463 ± 7.21	1313 ± 4.72	1575 ± 7.0	1672 ± 10.96	1422 ± 7.21	1391 ± 7.76	1491 ± 6.08	1605 ± 7.23	1318 ± 7.0	1407 ± 8.18
DNB	0.32	37 ± 1.52	58 ± 3.05	25 ± 1	22 ± 2	369 ± 9.0	375 ± 7.37	21 ± 2.08	24 ± 2.08	269 ± 3.60	279 ± 9.07	45 ± 6.0	46 ± 1.52
DNB	1.6	29 ± 1.73	59 ± 10.21	21 ± 1	20 ± 2.08	422 ± 7.0	423 ± 8.14	25 ± 1.52	26 ± 1.52	281 ± .5.03	289 ± 3.05	46 ± 2.3	433 ± 1.0
DNB	8	44 ± 4.04	46 ± 2.0	20 ± 1.52	19 ± 1.52	442 ± 6.7	451 ± 10.01	31 ± 1.0	55 ± 3.21	284 ± 3.6	305 ± 4.0	39 ± 1.52	43 ± 2.08
DNB	40	34 ± 0.57	45 ± 1.15	21 ± 1	20 ± 1.52	**476 ± 5.56**	**493 ± 5.29**	**46 ± 2.0**	**106 ± 4.35**	**298 ± 3.0**	**349 ± 5.03**	37 ± 2.64	37 ± 1.73
DNB	200	19 ± 3.46	43 ± 3.78	18 ± 1.52	20 ± 1	**496 ± 5.68**	**520 ± 7.54**	**54 ± 4.58**	**122 ± 7.02**	**304 ± 4.16**	**428 ± 5.29**	36 ± 3.05	39 ± 1.52
DNB	1000	20 ± 2.0	33 ± 2.51	17 ± 0.57	20 ± 1	**530 ± 6.65**	**539 ± 5.13**	**262 ± 4.58**	**196 ± 5.13**	**317 ± 3.78**	**579 ± 8.73**	33 ± 2.08	35 ± 3.05
DNB	5000	17 ± 1.52	25 ± 3.51	16 ± 1.15	16 ± 1.52	**560 ± 4.04**	**593 ± 4.35**	**344 ± 7.57**	**350 ± 6.24**	**419 ± 3.78**	**598 ± 3.0**	27 ± 2.08	28 ± 0.57

3,5-dinitro-N-(1-(p-tolyl)ethyl)benzamide(DNB analog) that cause base pair substitution mutation primarily at one of the GC pairs. In TA100 Mutagenic index increases (1.66, 1.90, 1.99, 2.14, 2.23, 2.39, 2.52 without-S9) and (1.55, 1.75, 1.86, 2.04, 2.15, 2.23, 2.45 with-S9) at 0.32, 1.6, 8, 40, 200, 1000 and 5000 µg/plate, respectively.

In strain TA1535 mutagenic index increases (1.01, 1.2, 1.47, 2.19, 2.57, 12.47, 16.39, without-S9) and (1.01, 1.11, 2.31, 4.41, 5.09, 8.19, 14.58 with-S9) at 0.32, 1.6, 8, 40, 200, 1000 and 5000 µg/ plate, respectively. In contrast, TA102 contain hisG428 mutation site that contain AT base pair at primary reversion site showed sign of mutagenicity. Mutagenicity, more specifically with metabolic activation at 1000 and 5000 µg/plate (1.01, 1.05, 1.05, 1.11, 1.13, 1.18, 1.56 without-S9) and (0.95, 0.98, 1.04, 1.19, 1.46, 1.97, 2.04 with-S9) as depicted in Table 3.

Mutagenicity in frameshift mutation sensitive *S. typhimurium* strains

Results, in the form of mutagenic index depicted in Table 4, shows no reversion to wild type was observed after treatment with anti-tuberculosis compound (R)-3,5-dinitro-N-(1-(p-tolyl)ethyl) benzamide(DNB analog) without(-S9) and with(+S9) metabolic activation in frame shift mutation sensitive *S. typhimurium* TA98, TA1537, carrying hisD3052 and hisC3076 mutation.

Comet assay for determination of DNA damage

Comet assay under neutral electrophoresis conditions was performed to examine nuclear DNA integrities, so that DNA double-strand breaks could be detected. DNA strand breakages in HL-60 cells were analyzed after exposure to 0.22 mM and 0.04 mM of anti-tuberculosis compound (R)-3,5-dinitro-N-(1-(p-tolyl)ethyl) benzamide(DNB analog) for 48 hours. The image of representative nuclei after electrophoresis of test compound treated cells showed formation of typical comets, with Head DNA of 60.81% and 65.21% at 0.22 and 0.04 mM respectively. Tail DNA which reveals the actual DNA damage was found to be 39.19% and 34.79% at the respective concentrations. The Olive tail movement was 2.14% and 2.34%. Tail length was found to be 4.67% and 4.41% respectively (Table 1). Camptothecin (5 µM) taken as a positive control showed a head DNA of 65.21% and a Tail DNA of 34.79%. However, no comet formation was observed in untreated cells.

Cytotoxicity assay

Cytotoxic effect of (R)-3, 5-dinitro-N-(1-(p-tolyl)ethyl) benzamide(DNB analog) have been determined on liver derived HepG2 cells at various concentrations for 48 hrs using MTT assay. Results showed that Anti-TB DNB analog found safe at lower concentration, however growth inhibition observed in concentration dependent manner (Figures 1 and 2), maximum 40% Growth Inhibition (GI) /inhibition of proliferation was observed at higher conc. (10 mM).

Discussion

In this study (R)-3,5-dinitro-N-(1-(p-tolyl)ethyl)benzamide, our potent anti-TB compound active against sensitive strain (H37rV) and resistant strains of Mycobacterium tuberculosis (Mungala et al. 2013) was evaluated for mutagenic potential.

This dinitrobenzamide (DNB) analog detected to induce base pair substitution specific mutation in *S. typhimurium* strains (TA100, TA1535, TA102) carrying mutation in hisG gene (hisG46, hisG428) of histidine operon (Table 3). Other hand no mutagenic activity was observed in *E. coli WP2uvrA* carrying mutation in gene (trpE) for biosynthesis of tryptophan specifically sensitive for base pair substitution (Table 4). No dose dependent increase of revertants was observed in frameshift mutation specific strains (TA98, TA1537) in AMES bacterial reverse mutation assay in the absence (-S9) and presence (+S9) of metabolic activation system (Table 4). Subsequently DNA damage was observed in comet assay (Figure 1) and (Table 5) shows high prediction of mutagen by *Salmonella, E. coli* bacterial reverse mutation assay.

In one of the well cited study conducted by Barnes et al. [21], they had reported base sequence analysis of His+ revertants of the hisG46 missense in *S. typhimurium* and this hisG46 marker in strains TA1535

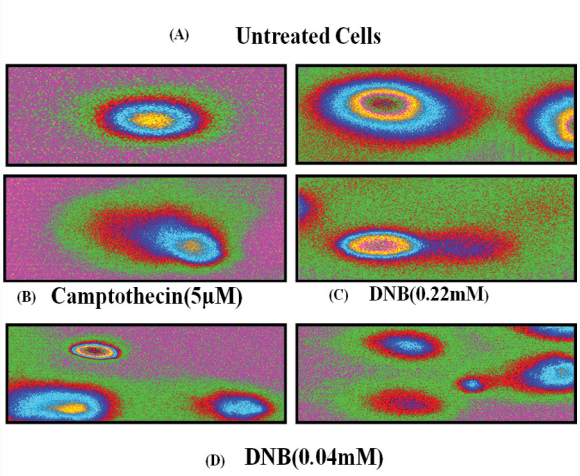


Figure 1: Comet formation in DNB treated HL-60 cells. (A) Untreated cells. (B) Camptothecin (5 μM) taken as a positive control. (C) DNB treated cells for 48 h of incubation.

and TA100 results from the substitution of a leucine (GAG/CTC) by a proline (GGG/CCC), further author reported that this mutation is reverted to the wild-type state by mutagens that cause base-pair substitution mutations primarily at one of the GC pairs. Our results also explains, that DNB analog reverting TA100 and TA1535 strains in wild type and regain biosynthesis of histidine in the absence and presence metabolic activation as indicated by high mutagenic index increased in dose dependent manner (Table 3) compared to TA102 where mutagenic effect was after metabolic activation (Table 3), these data clearly indicate that (R)-3,5-dinitro-N-(1-(p-tolyl)ethyl) benzamide specifically act on hisG gene of histidine operon, more specifically hisG46 irrespective of metabolic activation in TA1535 andTA100.

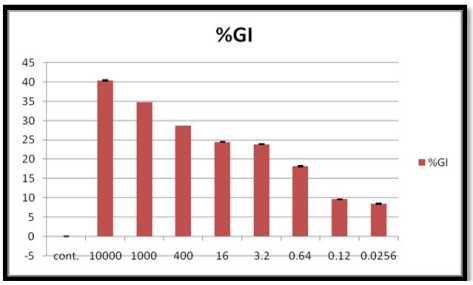
In addition to above the different pattern of the mutagenicity of DNB analog in strain TA102 (mutagenicity after metabolic activation) might be due to it carrying different type of mutation compare to TA1537, TA100 like; AT base pair at primary reversion site, hisG428 mutation is a ochre mutation, TAA, in the hisG gene which can be reverted by all six possible base pair changes; both transition and transversions, multicopy plasmid pAQ1, DNA repair proficiency (Table 1) as described in detail earlier by Levin et al. [23], Mortelmans and Zeiger [11].

Surprisingly, in this study, results (Table 3) of the treatment of DNB analog to *E. coli WP2uvrA* (non mutagenic) was found to be opposite compared to *S. typhimurium* TA102 (mutagenic), although OECD recommends either *S. typhimurium* TA102 or *E. coli WP2uvrA* in battery of bacterial strain for detection of mutagen in bacterial reverse mutation test (OECD471). It is known that both have an AT base pair at primary reversion site and specifically included in battery to detect certain oxidizing mutagen, cross-linking agents and hydrazines (OECD471) and earlier both the strain was compared in detail by Wilcox et al. [27]. It is also known that both strain differ on gene carrying mutation site; hisG428 of histidine operon (TA102) and tryE of tryptophan operon [23].

Interestingly DNB analog, does not shows its mutagenic activity in strain TA98, TA1537. These strains are known to carrying hisD3052 mutation (TA98), that is a -1 frameshift mutation which affects the reading frame of a nearby repetitive –C–G–C–G–C–G–C–G– sequence [24,28-30]. Earlier it was reported that reversion of the hisD3052 mutation back to the wild-type state is induced by various frameshift mutagens such as 2-nitrofluorene and various aromatic nitroso derivatives of amine carcinogens. Whereas strain TA1537 which carries the hisC3076 mutation as detected and reported earlier [1] to have a +1 frameshift mutation near the site of a repetitive –C–C–C– sequence and is reverted to the wild-type level by frameshift mutagens that are not readily detected by the hisD3052 marker, such as 9-aminoacridine [1,22] also shows non mutagenic in this study after treatment with DNB analog.

This study has investigated the DNA damage (Figure 1) and (Table 5), in single cell gel electrophoresis (comet assay) using HL60 cells, resulting in DNA fragmentation which migrated away from the nucleus giving a comet-like appearance (Figure 1) with the head consisting of intact DNA and the tail consisting of damaged DNA after the treatment with (R)-3,5-dinitro-N-(1-(p-tolyl)ethyl) benzamide(DNB analog) which shows DNB analog is DNA reactive and genotoxic (indicated by % tail DNA as depicted in Table 5.

Further, results of this study reveals, high degree of concordance between results of the AMES bacterial reverse mutation assay and comet assay, It is believed that compound positive in two *in vitro*



Conc in μg	Negative control	10000	1000	400	16	3.2	0.64	0.12	0.0256
GI%	$0_{\pm}$0.10	$40.39_{\pm}$ 0.10	$34.79_{\pm 0.02}$	28.73 +0.02	24.53+0 .07	23.88+0 .05	18.21+0. 20	9.66+ 0.05	8.45+0. 16

Figure 2: The cell proliferation assay of R)-3,5-dinitro-N-(1-(p-tolyl)ethyl)benzamide(DNB) in HepG2 cell by 48 hr treatment protocol, where it shows around 8.45% to 40% inhibition at the concentration of 0.0256 μg to 10 μg.

Table 3: Shows base pair substitution specific Mutagenicity in Salmonella typhimurium strains (TA100, TA1535, TA102, carrying mutation at hisG gene) showing post treatment (R)-3,5-dinitro-N-(1-(p-tolyl)ethyl)benzamide(DNB))dose dependent increase of mutagenic Index(MI) (MI=No. of revertants with treatment/ spontaneous revertants with vehicle control).

Mutagenic Index(MI) after treatment with DNB using *S. typhimurium*(hisG) and *E. coli WP2uvrA* strains specific for Base pair substitution									
		TA 100(HisG46)		**TA 1535(HisG46)**		**TA102(HisG428**		***E. coli WP2uvrA***	
Test Compound	Dose(µg/plate)	(- S9)	(+ S9)	(- S9)	(+ S9)	(- S9)	(+ S9)	(- S9)	(+ S9)
Vehicle control	0	1	1	1	1	1	1	1	1
Positive Control	+c	7.1	7.1	66.71	57.98	5.56	5.47	32.68	30.81
DNB	0.32	1.66	1.55	1.01	1.01	1	0.95	1.11	1.01
	1.6	1.9	1.75	1.2	1.11	1.05	0.98	1.15	0.94
	8	1.99	1.86	1.47	2.31	1.05	1.04	0.97	0.94
	40	2.14	2.04	2.19	4.41	1.11	1.19	0.91	0.81
	200	2.23	2.15	2.57	5.09	1.13	1.4	0.9	0.86
	1000	2.39	2.23	12.47	8.19	1.18	1.97	0.82	0.78
	5000	2.52	2.45	16.39	14.58	1.56	2.04	0.67	0.62

Table 4: Shows non mutagenic activity of (R)-3,5-dinitro-N-(1-(p-tolyl) ethyl) benzamide(DNB). Using Frame shift mutation specific *Salmonella typhimurium* strains TA98 (hisD3052), TA1537 (hisC3076) at various concentration in Bacterial reverse gene mutation assay as no dose dependent increase of Mutagenic Index (MI).

Mutagenic Index(MI) after treatment with DNB using *S. typhimurium* strains specific for frameshift mutation					
		TA98(hisD3052)		TA1537(hisC3076)	
Test Compound	Dose (µg/plate)	S9	S9	S9	S9
Neg. Control	0	1	1	1	1
Pos. Control	+c	10.62	3.05	69.66	52.54
DNB	0.32	0.82	1.03	1.19	0.88
	1.6	0.64	1.05	1	0.88
	8	0.97	0.82	0.96	0.78
	40	0.75	0.8	1	0.81
	200	0.42	0.76	0.88	0.8
	1000	0.44	0.58	0.84	0.8
	5000	0.35	0.41	0.77	0.66

Table 5: Table showing % distribution DNA (Head, tail), tail movement and length as results of the comet assay using DNB treated and untreated HL60 cells.

% Distribution of DNA				
Conc. of compound tested	**Head DNA**	**Tail DNA**	**Olive Tail Movement**	**Tail length**
Untrated Cells	98.78	1.22	0.09	0
Camtothecin(5µM)	65.21	34.79	7.35	35.14
DNB(0.22)mM	60.81	39.19	2.14	4.67
DNB(0.04)mM	65.21	34.79	2.34	4.41

assays; bacterial gene mutation and DNA damage in mammalian cell *in vitro*, greater the chances to be positive in *in vivo* mutagenic/ genotoxic assays and or rodent carcinogenicity.

Some studies indicate that different agents may act at different stages in the carcinogenic process and that several different mechanisms may be involved such as mutation in gene, DNA strand breaks, and formation of DNA adducts, chromosomal aberrations and aneuploidy etc.

Conclusion

Results of the mutagenicity study revealed that (R)-3,5-dinitro-N-(1-(p-tolyl)ethyl) benzamide (DNB) is a mutagenic anti-tuberculosis compound and causes mutagenicity only in base pair substitution specific *S. typhimurium* strain TA100, TA1535, TA102, carrying mutation in hisG gene of histidine operon. This study provides useful information on mutagenicity and genotoxic properties of the DNB indicates possible carcinogenicity by nitro group containing compounds and requirement of testing pharmaceuticals and chemicals for mutagenicity to save time and money involved in the process of drug development against tuberculosis.

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References

1. Ames BN, Lee FD, Durston WE. An improved bacterial test system for the detection and classification of mutagens and carcinogens. Proc Natl Acad Sci U S A. 1973;70(3):782-6.
2. Ames BN, McCann J. Detection of carcinogens as mutagens in the *Salmonella*/ microsome test: assay of 300 chemicals: discussion. Proc Natl Acad Sci U S A. 1976;73(3):950-4.
3. Mahon GAT, Green MHL, Middleton B, Mitchell IDG, Robinson WD, Tweats DJ. Analysis of data from microbial colony assays, in: Kirkland DJ, Editor. Statistical Evaluation of Mutagenicity Test Data, Cambridge University Press, Cambridge. 1989:28-65.
4. McCann J, Choi E, Yamasaki E, Ames BN. Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc Natl Acad Sci U S A. 1975;72(12):5135-9.
5. McCann J, Spingarn NE, Kobori j, Ames BN. Detection of carcinogen as mutagen: bacterial tester strains with R factor plasmids. Proc Natl Acad Sci U S A. 1975;72(3):979-83.
6. Sugimura T, Sato S, Nagao M. Overlapping of carcinogens and mutagens in: Magee PN, Takayama S, Sugimura T, Matsushima T, Editors. Fundamental of cancer prevention. University park press, Baltimore. 1976;191-215.
7. Zeiger E, Haseman JK, Shelby MD, Margolin BH, Fennant RW. Evaluation of four *in vitro* genetic toxicity tests for predicting rodent carcinogenicity; confirmation of earlier results with 41 additional chemicals. Environ Mol Mutagen. 1990;16(Suppl 18):1-14.
8. OECD. Guideline for testing of chemicals, Test Guideline 471: Bacterial Reverse Mutation Test. Organization for Economic Co-operation Development. France. 1997.
9. ICH. Guidance on specific aspects of regulatory genotoxicity tests for pharmaceuticals, international commission on harmonization of technical requirement for registration of pharmaceuticals for human use. International Commission on Harmonization. 1995.
10. Toxicological principle for the safety assessment of direct food additives and colour additives used in food "Readbook II" (Draft). FDA Center for Food Safety Applied Nutrition, Washington DC. 1993.
11. Mortelmans K, Zeiger E. The Ames *Salmonella*/microsome mutagenicity assay. Mutat Res. 2000;455(1-2):29-60.
12. Stover CK, Warrener P, VanDevanter DR, Sherman DR, Arain TM, Langhorne MH, et al. A small-molecule nitroimidazopyran drug candidate for the treatment of

tuberculosis. Nature. 2000;405(6789):962-6.

13. Ginsberg A, Spigelman M. New drugs for tuberculosis. In: Reichman L, Hershfield E, editors. Tuberculosis: a comprehensive international approach.3rd ed. New York and London: Informa Healthcare. 2006:1135-51.

14. Spigelman MK. Current and future therapeutic options for tuberculosis [presentation 2190 in symposium 220{F}]. In: Program and abstracts of the 45th Interscience Conference on Antimicrobial Agents and Chemotherapy. Washington, DC: American Society for Microbiology. 2005:115.

15. Munagala G, Yempalla KR, Aithagani SK, Kalia NP, Ali F, Ali I, et al. Synthesis and biological evaluation of substituted N-alkylphenyl-3,5-dinitrobenzamide analogs as anti-TB agents. Med Chem Commun. 2014;5(4):521-7.

16. Manjunatha UH, Boshoff H, Dowd CS, Zhang L, Albert TJ, Norton JE, et al. Identification of a nitroimidazo-oxazine-specific protein involved in PA-824 resistance in Mycobacterium tuberculosis. Proc Nat Acad Sci U S A. 2006;103(2):431-6.

17. Tyagi S, Nuermberger E, Yoshimatsu T, Williams K, Rosenthal I, Lounis N, et al. Bactericidal activity of the nitroimidazopyran PA-824 in the murine model of tuberculosis. Antimicrob Agents Chemother. 2005;49(6):2289-93.

18. Matsumoto M, Hshizume H, Tomishige T, Kawasaki M. *In vitro* and *in vivo* efficacy of novel antituberculous candidate OPC-67683 [abstract F-1462]. In: Program and abstracts of the 45th Interscience Conference on Antimicrobial Agents and Chemotherapy. Washington, DC: American Society f or Microbiology. 2005:204.

19. Tsubouchi H, Sasaki H, Haraguchi Y. Synthesis and antituberculous activity of a novel series of optically active 6-nitro-2,3-dihydroimidazol [2,1-b] oxazoles [abstract F-1473]. In: Program and abstracts of the 45th Interscience Conference on Antimicrobial Agents and Chemotherapy. Washington, DC: American Society f or Microbiology. 2005:207.

20. Kawasaki M, Yamamoto K, Matusmoto M. Mechanism of action of OPC-67683 against Mycobacterium tuberculosis [abstract F-1463]. In: Program and abstracts of the 45th Interscience Conference on Antimicrobial Agents and Chemotherapy. Washington, DC: American Society for Microbiology. 2005:204.

21. Hashizume H, Ohguro K, Itoh T. OPC-67683, a novel imidazooxazole antituberculous compound, free from mutagenicity: structureactivity-mutagenesity relationships [abstract F-1464]. In: Program and abstracts of t he 45th Interscience Conference on Antimicrobial Agents and Chemotherapy. Washington, DC: American Society for Microbiology. 2005:204.

22. Barnes W, Tuley E, Eisenstadt E. Base-sequence analysis of HisC revertants of the hiG46 missense mutation in *Salmonella* typhimurium. Environ. Mutagen. 1982;4:297(abstract Aa-1).

23. Levin DE, Yamasaki E, Ames BN. A new *Salmonella* tester strain for the detection of frameshift mutagens: a run of cytosines as a mutational hot-spot. Mutat Res. 1982;94(2):315-30.

24. Levin DE, Hollstein MC, Christman MF, Schwiers EA, Ames BN. A new *Salmonella* tester strain (TA102) with A: T base pairs at the site of mutation detects oxidative mutagens. Proc Natl Acad Sci U S A. 1982;79(23):7445-9.

25. Isono K, Yourno J. Chemical carcinogens as frameshift mutagens: *Salmonella* DNA sequence sensitive to mutagenesis by polycyclic carcinogens. Proc Natl Acad Sci U S A. 1974;71(5):1612-7.

26. Ahmad L, Jalali S, Shami SA, Akram Z. Sperm Preparation: DNA Damage by Comet Assay in Normo- and Teratozoospermics. Arch Androl. 2007;53(6):325-38.

27. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res. 1988;175(1):184-91.

28. Wilcox P, Naidoo A, Wedd DJ, Gatehouse DG. Comparison of *Salmonella typhimurium* TA102 with Escherichia coli WP2 tester strains. Mutagenesis. 1990;5(3):285-291.

29. Witte I, Plappert U, De WH, Hartmann A. Genetic toxicity assessment: employing the best science for human safety evaluation part III: the comet assay as an alternative to *in vitro* clastogenicity tests for early drug candidate selection. Toxicol Sci. 2007;97(1):21-6.

30. Bhushan S, Kumar A, Malik F, Andotra SS, Sethi VK, Kaur IP, et al. A triterpenediol from Boswellia serrata induces apoptosis through both the intrinsic and extrinsic pathways in human leukemia HL-60 cells. Apoptosis. 2007;12(10):1911-26.