Tropical Journal of Pharmaceutical Research November 2020; 19 (11): 2369-2375
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v19i11.18

Original Research Article

In-vivo antitrypanosomal effect and *in-silico* prediction of chronic toxicity of N-methylholaphyllamine in rats

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Sent for review: 7 June 2020 *Revised accepted: 20 October 2020*

Abstract

Purpose: *To determine the in-vivo anti-trypanosomal effect and sub-chronic toxicity of N-methylholaphyllamine (MHA) isolated from H. africana against Trypanosoma brucei in rats and also to predict its toxicity by an in-silico method.*
Methods: *Parasitemia was induced in rats with 1.5 x 10^5/mL trypanosomes and treatment commenced 5 days post-infection for 12 days. The rats were treated with MHA (3.5 µM/rat) for 5 days and with diminazene (3.5 mg/kg) for 2 days and were monitored every other day during and after treatment for the level of parasitemia and PCV. The chronic toxicity study was carried out with a 28-day sub-chronic toxicity cycle protocol while the toxicity was predicted in-silico with ProTox-II which is freely available on a web server.*
Results: *MHA exhibited anti-trypanosomal effect in infected rats leading to the restoration of PCV to baseline values (≥ 40 %) on the 14th day and consequent disappearance of parasitemia on day 17 post-infection with no relapse. The slight changes in clinical observation, weight, feed consumption, clinical and histopathology of high-dose MHA rats were not significant ($p < 0.05$) and were not attributed to the treatment. Apart from MHA-induced immunotoxicity observed in in-silico prediction, no other predicted toxicities were significant; however, few undetected toxicities were found to be mediated by amine oxidase A, androgen and/or histamine, H1 receptors toxicophore fit.*
Conclusion: *The high in-vivo antitrypanosomal effect and non-toxicity of MHA in this study further provide useful empirical data for lead optimization of MHA to combat sleeping sickness.*

Keywords: *Holaphyllamine, Antitrypanosomal, Trypanosomes, Chronic toxicity, In-silico*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Apart from pharmacokinetics-related problems, many drug discovery failures can be attributed to the efficacy and toxicity profiles of molecules. Toxicity measurement depends on several factors such as exposure time (acute or chronic), administered dose and the target affected [1]. The liver and kidney are the primary organs for bioaccumulation of toxicants and they play significant roles in the elimination, biotransformation, storage and redistribution of toxicants in the body [2,3]. The global burden of organ toxicities affects over 100 million people

worldwide [4].

No compound- natural or synthetic- is free of toxicity; however, benefit-to-risk assessment (BRA) is considered paramount throughout the life cycle of drug discovery [5]. Such assessment allows the selection of molecules with the best BRA for lead optimization and candidate selection [6] Many alkaloids, including the recently identified anti-trypanosomal steroids from *H. africana* could be sufficiently toxic to animals to cause death when consumed [7,8]. Studies had shown that some steroidal alkaloids from *Solanum tuberosum* cause embryo mortality and other forms of toxicities in animals [9]. The steroid alkaloids-α-chaconine, α-solanine, solanidine and solasodine- originally isolated from *Solanum tuberosum* possess a basic cyclopentanophenanthrene skeleton similar to the holaphyllamines. Previous reports identified holaphyllamine and several of its analogues as hits against African trypanosomiasis [7]. To further optimize them as a lead against African trypanosomiasis, *in-vivo* effects as well as toxicity studies are imperative. N-methylholaphyllamine (MHA) was found to be the major constituents of the leaves of *H. africana* (Family Apocynaceae) and also sufficiently trypanocidal in the *in-vitro* testing against *T. brucei* species. Due to ethical issues, time and cost which limit animal studies, *in-silico* method is a highly evolving alternative to complement *in-vitro* methods of toxicity testing [10]. One of such *in-silico* methods is the PRediction Of TOXicity (ProTox) webserver which is freely available, incorporates both chemical and molecular targets, provides insights into the possible mechanism of toxicity response and also provides different levels of toxicity such as oral toxicity, organ toxicity, toxicological endpoints, toxicity targets and toxicological pathways [11]. Against these backdrops, this study presented the *in-vivo* trypanocidal effects and sub-chronic toxicity in the animal model as well as the prediction of the toxicological profile of this important representative of the holaphyllamine compounds.

EXPERIMENTAL

Induction of parasitemia

A 1 mL blood of rat infected with *T. brucei* species, collected through the media cantus of the eye, was diluted with 9 ml of normal saline and 0.2 mL (containing 1.5 x 10^5/mL trypanosomes) administered i.p to the rats. The rats were fed *ad libitium* with free access to clean water and monitored for 5 days until the level of parasitemia was significant (> 10^7 trypanosomes/mL), after which baseline parasitemia level and PCV were determined. The level of infection was ascertained in each rat tail blood examination for trypanosomes using a rapid matching counting method [12].

In-vivo animal studies

The permission to use animals for this study was obtained from the University of Nigeria Ethics Committee on the handling of laboratory animals (approval no. IEC/FVM/19/010). All animal treatments were performed in line with international guidelines of the National Institutes of Health publication no 85-23, revised 1985 [13]. The rats were divided into four (n=5) groups as follows: Group 1 was infected and treated with an optimized dose of 3.50 µM/rat (equivalent to 2 times *in-vitro* IC_{50}) of MHA in Tween 80 for 5 days. Group 2 was infected and treated with 3.5 mg/kg of diminazene for 2 days. Group 3 was infected but not treated (5 mL/kg normal saline) while Group 4 served as the uninfected and untreated control. The samples were dissolved in Tween 80 and administered orally. All treatments started 5 days post-infection and lasted for 12 days. The rats were monitored every other day during and after treatment for the level of parasitemia and PCV.

Chronic toxicity study

In this study, 15 healthy albino rats (weighing 180 ± 2 g) of either sex were divided into three groups of 5 rats/group. The toxic dose of MHA was determined following the previous protocol [3]. A 0.8 mL oral dose (8.75 µM in Tween 80) of MHA, 0.8 mL Tween 80 and 0.80 mL chloroform/ether (v/v) as control were selected for the study and designated groups A, B and C respectively and administered to the rats for 30 consecutive days. The rats were observed daily for some visible signs and weekly for changes in feed intake and body weight. Thereafter, blood was collected for hematological and biochemical tests and the liver was used for histopathological examinations. In all cases, the first day of dosing was day 1 of the study and dosing was at the same time each day. Animals were allowed free access to feed and water throughout the study. However, animals were fasted overnight before blood collection on the 29th day and before organ harvest on the 31st day of the study [14].

Signs of toxicity study

Some signs of toxicity and abnormal behaviours were recorded for all the animals on day 1 before the administration of the samples and every week subsequently. Detailed signs such as

mortality, aberrant behaviours, stereotypies, autonomic activities were recorded while handling the animals as well as in the open field.

Determination of body weight

The animals were weighed during acclimatization and on day 1 of the treatment. Subsequently, weighing was done weekly and weight gain or loss was calculated at each time interval throughout the study.

Assessment of feed intake

Rats were allowed access to feed and feed consumption was measured every week. The mean feed intake was calculated for the weekly intervals and the overall testing period.

Determination of clinical-pathological parameters

Before this study, the rats fasted overnight and blood samples were collected through orbital sinus bleeding on the study day 29 with a sterile syringe using heparin as an anticoagulant. A portion of the blood sample was centrifuged at 1000 g for 5 min to separate the plasma and the non-hemolyzed plasma was maintained at -20 ºC for biochemical analyses. The other portion of the blood sample was used to estimate blood count. Red blood cell (RBC), white blood cell (WBC) counts, PCV and haemoglobin (Hb), as well as WBC differentials, were determined using a standard protocol [3]. The sample used for the assay of hematological parameters was utilized for the determination of liver enzymes such as aspartate aminotransferase (AST) and alkaline transferase (ALT) and alkaline phosphatase (ALP). Renal activity such as urea, total protein (TP) and creatinine were also determined [3].

Histopathological examination of liver

On day 31 of the study, the rats were sacrificed and the liver harvested for organ weight determination and histopathological examination. The samples were fixed in 10 % phosphate-buffered formalin for 48 h before tissue preparation. The tissues were subsequently trimmed, dehydrated in four ascending grades of alcohol (70, 80, 90 and 100 %), cleared in three grades of xylene and embedded in molten wax. After embedding, the tissue-containing wax blocks were cut into 5µm thick sections with a rotary microtome, floated in the water at 60 ˚C, placed on clean grease-free glass slides and placed on a slide warmer set at 60˚C over-night. The 5 µm-thick sectioned tissues on glass slides were subsequently cleared in three grades of xylene and rehydrated in 3 descending grades of alcohol (90, 80 and 70 %). The sections were then stained with Mayer's hematoxylin for 5 min. Blueing was done with NH_4Cl and differentiation with 1 % acid alcohol before counterstaining with Eosin [15]. Permanent mounts were made on degreased glass slides using a mountant, DPX. The prepared slides were examined with a Motic™ compound light microscope using x4, x10 and x40 objective lenses. The photomicrographs were taken using a Motic™ 5.0 megapixels microscope camera at x100 and x160 magnifications.

***In-silico* prediction of toxicity**

The toxicities of MHA and related holaphyllamines were predicted using ProTox-II, a freely available web server (http://tox.charite.de/protox_II/) for *in-silico* toxicity prediction. The 2D chemical structures of the compounds were converted to their SMILES and input into the ProTox-II graphical user interface for toxicity computation of the machine learning algorithms-based models which included acute (oral) and organ (liver) toxicities as well as toxicological endpoints, pathways and targets. Prediction types, training/test set compounds, cross-validation, descriptors and all methods used for the prediction were adopted from the webserver without modification as previously applied [10,11].

Statistical analysis

The experimental results were analyzed using GraphPadPrism v.5 software. Data are expressed as a mean ± standard deviation (SD) (n = 5). One-way analysis of variance (ANOVA) was done to test for the significant differences between the means of samples and control using Dunnett post-hoc's test. In all cases, $p < 0.05$ was considered to be significant.

RESULTS

***In-vivo* trypanocidal effects of MHA**

The effects MHA on PCV and parasitemia levels of rats infected with trypanosomes are shown in Figure 1. There was an initial decline in PCV of rats infected with trypanosomes before the commencement of treatment on day 5. MHA significantly improved and restored PCV to baseline on the 14th day. It also caused a significant ($p < 0.05$) decline in parasitemia on the 11th day compared with untreated groups. MHA, however, was not as effective as diminazene which cleared the parasites in the

blood and subsequently restored the PCV to baseline values within 5 days of treatment.

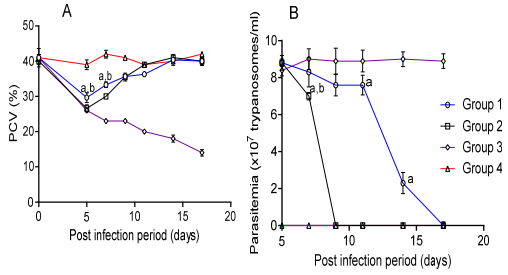

Figure 1: Effect of MHA on PCV (A) and parasitemia (B) of *T. brucei* infected rats; data expressed as mean ± SD (n = 5); $p < 0.05$ compared to [a]untreated and [b]diminazene-treated groups are considered significant

Chronic toxicity profile of MHA

There was no clinical observation of toxicological importance in MHA-treated rats (group A) or untreated group (group B) compared with the group treated with chloroform/ether (group C). Little aberrant behaviour in animals in group A such as walking backward and pupil size dilation were also observed in some animals in groups B when compared with stereotypies (excessive groaning) and autonomic behaviours (lacrimation) observed in group C animals. The mean body weights of all the rats in groups A and B increased within 1-28 days, however, there was no significant difference in body weights of group A compared with group B and could not be attributed to the treatment. Animals in group C showed significant weight reduction throughout the study period. The feed intake and feed efficiency of the MHA-treated group were similar to the untreated control group. However, an increase in food consumption was observed within a 22 - 28 days period in both groups A and B and, therefore, considered to be of no toxicological significance. However, feed intake in group C significantly decreased compared with group B.

Effect of MHA on clinical pathology

The effects of MHA on some hematological parameters and WBC differentials are shown in Table 1. There was no statistically significant difference in the hematological parameters of the MHA-dosed group when compared with the untreated. There was a significant reduction ($p < 0.05$) in WBC, RBC, PCV and Hb levels of chloroform/ether-treated group when compared with the untreated. A similar trend was observed in the results of the WBC differentials. Except for urea, there was no significant difference in the liver enzymes and differential of MHA-treated rats compared with untreated.

Effect of MHA on clinical histopathology of liver

To further examine the toxicity potential of MHA, a section of the liver was examined for damage. The images obtained for various treatments are shown in Figure 2. The liver sections of rats in groups A and B showed the normal hepatic histo-architecture while those of group C indicated widespread degenerated hepatocytes (degenerated central vein, hepatic vein and bile duct).

Table 1: Effect of MHA on some clinical-pathological parameters

Parameter	Group/treatment		
	A (MHA)	***B (Tween 80)***	***C (Toxicant)***
WBC x10^9 (mm^3)	5.93±0.42	6.30±0.44	6.87±0.30[a]
RBC x10^9 (L)	8.80±0.26	8.93±0.51	5.93±0.42 [a]
PVC (%)	41.0±0.74	40.80±1.65	23.60±0.48 [a]
HB (g/dL)	38.00±4.00[a]	34.00±4.36	27.00±4.58[a]
Neutrophil (%)	59.67±1.53	56.33±1.52	61.33±1.15[a]
Lymphocyte (%)	36.00±1.00	39.00±2.00	35.00±1.00[a]
Monocyte (%)	2.33±0.58	2.00±0.00	1.33±0.58 [a]
Eosinophil (%)	2.00±0.00	2.33±1.15	2.33±0.58
AST (u/L)	11.25±0.56	11.88±0.46	13.50±0 .47[a]
ALT (u/L)	11.13±0.27	11.74±0.49	13.50±0.52[a]
ALP (u/L)	61.03±2.06	62.53±3.63	82.63±5.34[a]
Creatinine	0.43±0.14	0.51±0.24	1.33±0.18[a]
Urea	33.33±5.27[a]	40.16±4.34	60.11±7.74[a]
Total protein	3.22±0.72	3.82±0.37	1.67±0.37[a]

Data are expressed as mean ± SD; [a]p < 0.05 compared with untreated

Table 2: *In-silico* predicted toxicities of holaphyllamines

Descriptor/compound	MHA	3α-DHA	3α-HA	3β-DHA	3β-HA
Average similarity (%)	77.91	95.24	80.96	95.24	80.96
Prediction accuracy (%)	69.26	72.9	70.97	72.9	70.97
Acute toxicity (mg/kg)	750.0	440.0	750.0	440.0	750.0
Organ toxicity (dili)	0.77	0.83	0.75	0.83	0.75
Toxicity endpoints (immuno)	0.68	0.52	0.97	0.52	0.97
Toxicity pathways	Inactive	Inactive	Inactive	Inactive	Inactive
Toxicity targets (%)	Andr (1.43)	0.0	0.43	0.0	0.43
	Aofa (45.88)	-	41.53	-	41.53
	Hrh1 (0 %)	-	Gcr (2.12)	-	Gcr (2.12)

Dihydroholaphyllamine (DHA), holaphyllamine (HA), drug-induced liver injury (dili), immunotoxicity (immuno), androgen receptor (andr), amine oxidase A (aofa), histamine receptor (hrH1), glucocorticoid receptor (gcr)

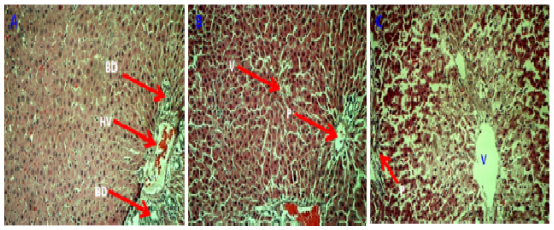

Figure 2: Histoarchitectural changes in liver sections of rats dosed with MHA (A), Tween 80 (B) and toxicants (C); hepatic vein (HV), bile duct (BD), central vein (V), portal area (P), H&E stained x160

In-silico toxicity prediction

The predicted median lethal dose (LD_{50}), toxicity class, toxicological pathways and targets information together with the name of the targets/pathways as well as the average fit and similarity of the input compound with the pharmacophore and known ligands of the respective targets are shown in Table 2. The toxicity radar plot which compared the average confidence score of the bio-active compounds in the training set of each model to that of MHA is shown in Figure 3. The prediction showed that the compounds showed similar toxicity profiles. The average similarity indicated >70 % of holaphyllamines to molecules that have been shown to bind at the modeled targets.

DISCUSSION

In a previous study, several steroid alkaloids, mostly holaphyllamine analogues isolated from *H. africana* were identified as hits in the search for compounds with antitrypanosomal effects [7]. The compounds were screened *in-vitro* for toxicity against mammalian cells. To correlate the *in-vitro* data with *in-vivo* effects, a representative of the holaphyllamine family was selected for further *in-vivo* studies. This study revealed an interesting trend in the effects of MHA against *T. brucei brucei*.

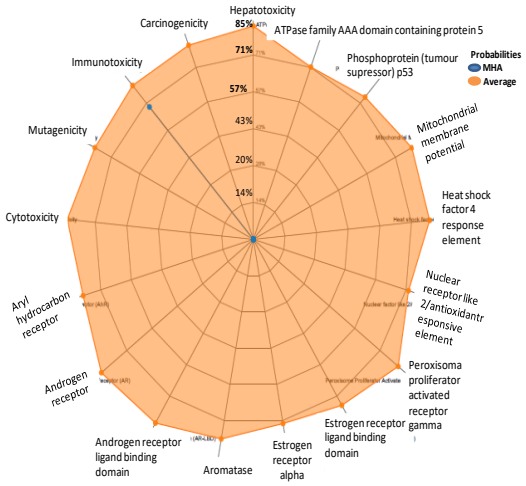

Figure 3: ProTox radar plot for MHA (*MHA is predicted to be active for one endpoint, connecting different layers of the ProTox-II classification scheme; orange dots/lines are the average probability of its active class, acquired by computing from the training set data for each model)*

Preliminary studies had suggested that the aqueous extract of this plant cleared the trypanosome by day 9 post-treatment in mice which remained aparasitemia until day 14 when relapse occurred [16]. This study showed similar trends in clearing the parasite; however, the parasite clearance was slightly slower beginning on day 11 until the 17th day post-infection with no relapse. These findings were in agreement with the slow onset of trypanocidal activity of these holaphyllamines in the *in-vitro* mechanistic studies reported elsewhere [17]. This slow onset of action is a potential beneficial property of the holaphyllamines that is also common with diamidines [18]. To complement the in-vivo effects on the parasites, MHA caused the restoration of the PCV of the infected rats to the baseline values from the 11th to 15th day post-infection. However, the restoration was also slower compared with the effect of diminazene aceturate.

The investigation of the toxicological properties of molecules is a crucial step in the drug development process. A high dose of MHA administered to rats for 28 days caused no significant toxicity attributable to the treatment. All the signs of toxicity recorded such as autonomic activities, changes in gait, posture, fur, eyes, pupil size, the occurrence of secretion or excretion, aberrant behaviour and stereotypic movements suggested no evidence of toxicological activities linkable to the treatments. The mean body weights and the feed consumption efficiency followed a similar trend in groups A and B. The surge in feed consumption by group A rats recorded from day 22 of the study was also observed in the untreated group B on day 25. The increased feed consumption in both groups was not significant to be attributed to MHA.

The clinical-pathological examinations showed slight changes in the hematological parameters and WBC differentials as well as the clinical chemistry parameters. However, no statistically significant difference was observed in the parameters of MHA-treated and untreated rats. The significant drop in PCV and Hb of the positive control group could be attributed to the altered physiological activities of rats, exhaustion of the immune system as well as immunosuppressive action of toxicants [3]. The low neutrophil and eosinophil as well as high lymphocyte and monocyte in rats treated with MHA compared with known toxicants provided further evidence of no hematological toxicity of MHA. The liver and kidney are major organs affected by the toxicity of drugs [3]. In cases of damage to the liver, certain enzymes are released in a large amount into the blood. These enzymes have always been used as non-invasive markers to detect organ damage to the liver. This study showed a surge in both plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the positive control group suggesting significant alterations in the enzyme activities in blood plasma due to widespread damage to the liver as previously reported [3,19].

The major kidney markers are urea, creatinine and total protein and elevated serum urea and creatinine indicate poor clearance of waste substances by the kidney as was observed in the toxicants-dosed rats. Such elevation is an indication of possible hepatocellular damage [20]. However, an elevation in total protein (obvious in the MHA-treated groups) signifies an increase in the body's immune protective ability [19]. To further confirm the non-toxicological effects of MHA on vital organs, the histopathological examination of the liver was conducted. There were no gross liver lesions in any of the rats from the MHA-treated and untreated groups. However, potential treatment-related histomorphological findings were limited to the chloroform/ether-treated group. Similar trends were seen in all rats in the group supporting a definitive association of the treatment and the severe-widespread degeneration and necrosis of the hepatocytes in the centrilobular to midzonal zones of the hepatic lobules observed in the liver of toxicant challenged rats. The study predicted the toxicity of MHA and related molecules using ProTox, a mechanism-based prediction and chemical toxicity evaluation tool [10]. For MHA, the nearly 70 % prediction accuracy with 77.9 % similarity with molecules sharing the same target showed robust prediction with no organ (liver) toxicity. In the toxicity endpoint model, immunogenicity was obtained with 68 % similarity to the 34086 immunotoxicants used as a training set for the model, however, no MHA-induced carcinogenicity, mutagenicity or cytotoxicity was observed. The predicted immunotoxicity could be attributed to the steroidal skeleton of MHA [8], Interestingly, MHA showed significant pharmacophore fit to androgen receptors (1.43 %) and amine oxidase A (45.99 %), however, all other toxicity pathways predicted in this study were inactive [11].

CONCLUSION

This study has shown that MHA is a slow-acting trypanocidal and non-toxic molecule in an in-vivo model. Furthermore, there is no evidence of treatment-related adverse events, thus, demonstrating that the tested MHA is non-toxic to rats with no overt adverse effect when dosed 8.75 µM/day orally for 28 consecutive days. The toxicity prediction gave an insight into a possible amine oxidase A and/or androgen receptor-mediated non-overt toxicity of holaphyllamines.

DECLARATIONS

Acknowledgement

The authors acknowledge Chinonye Igwe of the Department of Pharmaceutical Chemistry, UNN for providing facilities for toxicity studies and Ikechukwu Agbo of Veterinary Teaching Hospital, UNN for the donation of trypanosome culture.

Conflict of interest

Authors hereby declare that no conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities on claims relating to the content of this article will be borne by the authors. Wilfred Obonga, Charles Nnadi and Chinwe Onah supervised and analyzed the work. Linda-Mary Ozioko and Glory Eneje carried out the benchwork and drafted the manuscript while Wilfred Obonga and Charles Nnadi wrote the final version of the manuscript. Charles Nnadi developed the concept of this study and carried out the in-silico studies.

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