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Activity of MG-192 Against in Vitro Cultures of Infective Trypanosome Species and its Acute Toxicity Effects in Mice.

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Abstract

Trypanosomiasis is a disease caused by parasites of the *Trypanosoma* genus. The parasites are transmitted by the bite of an infected tsetse fly, resulting in human and animal disease. Trypanosomiasis is often fatal, unless chemotherapeutic intervention is sought.

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Treatment options are, however, few, complex to administer, with increasing cases of drug resistance being reported. Hence the need for development of a safer and more effective drug. A synthetic compound, code-named MG-192, was discovered to have similar stereo structure and functional groups as Pentamidine, an established drug used in the treatment of early-stage Trypanosoma brucei gambiense infections, as well as a few documented cases of T. b. rhodesiense disease. Using the structure-activity relationship theory, it was hypothesized that MG-192 has similar pharmacological activity to Pentamidine and can treat trypanosomal disease. Using in vitro assays, bloodstream forms of various infective trypanosomes were cultured in the presence of MG-192, in order to determine their sensitivity to MG-192. Acute toxic effects of MG-192 to mice systems were also studied, including body weight and packed cell volume changes. The average Minimum Inhibitory Concentration of MG-192 in in vitro cultures of T. brucei rhodesiense species KETRI 2537 and KETRI 2538 were 2.345 mg/ml ± 1.355 and 3.125 mg/ml respectively. For T. congolense isolates KETRI 3867 and KETRI 3805, the MIC of MG-192 was 3.125 mg/ml and 3.516 mg/ml±0.552 respectively. The No observable adverse effects level (NOAEL) of MG-192 administered intraperitoneally was 1000 mg/kg. There was a significant difference in the body weight and packed cell volume of mice administered with a single dose of MG-192 (750 mg/kg and 500 mg/kg), when compared to parameters of the control mice. The study concludes that MG-192 is effective in clearing T. b. rhodesiense and T. congolense bloodstream forms in vitro and has minimal toxicity effects on mice' weight and packed cell volume.

Keywords: Trypanosomiasis; structure-activity relationship theory; MG-192; drug development; in vitro efficacy; acute toxicity.

1. Introduction

Trypanosomiasis is a vector-borne disease caused by parasites of the Trypanosoma genus. In Africa, the disease is considered endemic in 36 countries, with 21 million people at a high risk of getting infected [1].The trypanosomes are transmitted through the bite of an infected Glossina spp. (tsetse) fly and they cause disease in both humans (human African trypanosomiasis- HAT) and animals (African animal trypanosomiasis-AAT). Trypanosoma brucei rhodesiense and T. brucei gambiense are the main causative agents of HAT, with T. b. rhodesiense resulting in acute disease and T. b. gambiense causing a more chronic form of the disease. African animal trypanosomiasis (AAT) causes disease in almost all domestic animals, leading to decreased livestock productivity and reduced socio-economic power in communities that rely heavily on agro-pastoralism [2]. Trypanosomiasis infections result in debilitating disease and eventual death if medical intervention is not sought. Chemotherapy therefore remains an important tool in controlling trypanosomiasis. This however, has its major setbacks such as the development of parasite resistance to the drugs, the cost of production, overt toxicity in patients and complex administration [3-4]. These factors have impeded their effectiveness in fighting the parasitic disease. It is therefore of great importance to continue research aimed at the development of novel, more efficacious, less toxic and cost effective drugs.

Recently, a synthetic compound code-named MG-192 was discovered to have similar structural and functional groups as those of Pentamidine, a WHO-approved therapeutic drug for treatment of early stage Trypanosoma brucei gambiense disease. MG-192 is an alcohol-based liquid, with an overall positive charge and an oily

composition. The concentration of MG-192, according to the manufacturer, is 10,000 mg/kg. The structure – activity relationship theory used in drug design states that the biological properties of a compound can be inferred from its structural characteristics, based on the assumption that similar compounds (or molecules) have similar chemical or biological properties [5]. It is in following with this theory that the hypothesis was developed to test the potential of MG-192 to treat trypanosomal infections.

2. Materials and Methods

The study was carried out at the Biotechnology Research Institute (formerly TRC-KARI) of Kenya Agricultural Livestock and Research Organization, at Muguga, Central Kenya.

2.1 Experimental Mice

Swiss white mice were purchased from the Biotechnology Research Institute's (BRI) small animal unit. All mice used were male, 6 weeks old and weighing 20 to 30 grams (g). The mice were housed in cages lined with wood chippings as bedding and fed on commercial mice pellets (Unga Ltd, Kenya) and water ad libitum.

2.2 Trypanosome strains

Infective trypanosomes were selected from the Institution's biobank, as follows;

KETRI 2537, KETRI 2538 for T. b. rhodesiense: KETRI 2537 is an isolate derived from EATRO 1989 and has been serially passaged in monkeys [6]. It is sensitive to diminazene aceturate and resistant to suramin in mice systems [7]. KETRI 2538 is an isolate that is melarsoprol- resistant [8].

KETRI 3867 and KETRI 3805 for T. congolense: KETRI 3867 is a field isolate, isolated from cattle in Njorore in coastal area of Kenya. The little-studied isolate is diminazene aceturate-sensitive in vitro [Unpublished data, Pharmacology Division of BRI]. KETRI 3805 is a clone of the isolate IL 3330 and exhibits resistance to multiple drugs in vitro and in vivo [9].

KETRI 3953, KETRI 3954, KETRI 3958, KETRI 3962, KETRI 3963 and KETRI 3966 for T. b. gambiense: These were all isolated from HAT-infected patients in South Sudan [10].

2.3 Drugs and Drug preparation

The test compound, MG-192, was in liquid form and came from the manufacturer in the concentration of 10,000 mg/kg. The compound was diluted to a working solution of 5,000 mg/kg by adding 100 ml phosphate saline glucose (PSG) solution to 100 ml MG-192 and gently shaking in order to homogenize it. The working solution was then stored in aliquots in dark bottles at room temperature and used within a week. All other drugs, Diminazene aceturate [(Veriben®,Ceva, France) Isometamidium chloride (Samorin®, Merial, France) for T. congolense isolates, Suramin (Germanin®- Bayer, Germany) Mel B (Arsobal®- Aventis, Germany) for T. brucei rhodesiense isolates and Pentamidine (Pentacarinat®-Sanofi, UK) for T. b. gambiense isolates] except

Mel B, were prepared by dissolving them in distilled water in accordance with the manufacturer's instructions. Mel B was constituted by diluting the drug using propylene glycol.

2.4 In vitro sensitivity assay

Donor mice were infected with cryopreserved trypanosome stabilates diluted in phosphate saline glucose (PSG). Donor mice to be exposed to T. b. gambiense were first immunosuppressed using a modified method described by [9]. Briefly, the mice were injected with 4 daily doses of 300 mg/kg cyclophosphamide (Acros Organics, New Jersey, USA) prior to inoculation with the parasites, and a single injection every 10 days post infection. Immunosuppression was done to increase the parasite levels in the mice blood. The mice were monitored for parasitaemia by observing a drop of blood from their tails under a light microscope (x400 magnification). When the parasitaemia peaked (antilog 8.1, based on the matching method by [10]), the trypanosomes were harvested and isolated from the mice blood. In vitro drug sensitivity determination was carried out in 96- well culture plates. A culture medium of Iscoves' modified dulbecco's medium (IMDM) containing soybean lipid, albumin 3.042 g/l sodium bicarbonate and transferrin was supplemented with 25 mM Hepes buffer. The medium was further enriched with 0.2 mM 2-mecarptoethanol, 2 mM sodium pyruvate, 0.002 mM bathocuproine sulfonate, 1.5 mM L-cysteine, 0.016 mM thymidine, 5 U penstrep solution and 20% inactivated fetal bovine serum, a modification of [11]. 100 ml of the culture medium was pipetted to each of the wells in the culture plate, except for the border wells, which were left unused due to the likelihood of evaporation of liquids from them.

Approximately 200 µL of various concentrations of the test compound (MG-192) were then added in duplicate rows in column 11, after which serial dilution was carried out by transferring 100 µl of the solution in well 11 to well 9, and so forth until well 4. From the wells of column 4, 100 µL solution was discarded. Columns 2 and 3 served as positive control wells containing no drug. To all the used wells, 100 µl of trypanosome suspension was added, with a seeding density of 1 x 10^5 trypanosomes/ml. Respective reference drugs were also plated for each isolate. The T. b. rhodesiense cultures were incubated in a 37^0C, 5 % carbon dioxide (CO^2) humidified environment for 72 hours (h), while the T. congolense cultures were incubated at 34^0C for 72 h. After incubation, the culture plates were observed under an inverted microscope at X 100 magnification. In every row, the highest dilution with no mobile trypanosomes was determined and this concentration was taken as the minimum inhibitory concentration (MIC).

2.5 Acute toxicity testing in mice

The mice used in this experiment were first dewormed with an intraperitoneal (i.p.) injection of 0.01 ml Ivermectin (Noromectin®- Norbrook), to rid of endoparasites and ectoparasites. The mice were left to acclimatize for 14 days, after which they were randomly placed in groups of 6 per cage [12].

Baseline parameters of the mice' weight and packed cell volume (PCV) were recorded: The PCV determination used was as described by [13], after which single toxicity studies commenced: The mice groups were given single i.p. injections of 5,000 mg/kg, 1000 mg/kg, 750 mg/kg, 500 mg/kg and 100 mg/kg. The control group was injected with 0.2 ml of phosphate glucose saline solution (PSG). The mice were then observed

immediately, after 15 minutes, then after one hour, two hours, four hours and every 24 hours thereafter for 14 days for any observable signs of overt toxicity. Their weights and PCV were also read twice a week for the duration of the observation. Mice showing adverse effects were immediately withdrawn from the study and euthanized.

3. Results

3.1. *In vitro sensitivity assay*

The effect of the drugs on the bloodstream forms of the trypanosomes in 96- well plates was assessed by culturing them in growth medium, to which the drugs were incorporated. Melasorprol (Mel B), a drug used to treat second-stage HAT infections, proved to be the most potent in preventing the replication of the T. b. rhodesiense parasites, by having a low MIC value (22.916 ng/ml ±3.579) (Table 1). Suramin was less potent, inhibiting/ killing the parasites with 13.54 µg/ml (±6.505) and 9.373 µg/ml (±5.63) in the KETRI 2537 and KETRI 2538 species respectively. The test compound, MG-192, showed the least potency, by inhibiting MIC growth in the range of 2.345 mg/ml and 3.125 mg/ml for the same Rhodesiense species. The T. congolense reference compounds, diminazene aceturate and isometamidium chloride (ISMM), had MIC values of 57.3 µg/ml (±9.007) and 62.5 µg/ml respectively, when cultured with a known resistant isolate; isolate KETRI 3805. The MIC value of MG-192 against the same isolate was 3.125 mg/ml. Against KETRI 3867, diminazene had a lower MIC of 0.695 µg/ml and ISMM, 2.60 µg/ml (±1.193). MG-192 also had a lower MIC of 3.125 mg/ml, compared to isolate KETRI 3805.

Table 1: In vitro sensitivity of various trypanosome species to test compounds

Isolate I.D	**Drug**	**MIC of test triplicate (± Std. Dev)**
T. b. rhodesiense KETRI 2537	Mel B	22.916 ng/ml ± 3.579
	Suramin	13.54 µg/ml ± 6.505
	MG-192	2.345 mg/ml ± 1.355
T. b. rhodesiense KETRI 2538	Mel B	22.933 ng/ml ± 3.579
	Suramin	9.373 µg/ml ± 5.63
	MG-192	3.125 mg/ml
T. congolense KETRI 3805	Diminazene	57.3 µg/ml ± 9.007
	ISMM	62. 5 µg/ml
	MG-192	3.516 mg/ml ± 0.552
T. congolense KETRI 3867	Diminazene	0.695 µg/ml
	ISMM	2.60 µg/ml ± 1.193
	MG-192	3.125 mg/ml

3.2 Acute Toxicity of MG-192 when administered to mice

In the mice group administered with 5000 mg/kg, 1 out of the 6 mice died instantly while two other mice in the

treatment group developed instant paralysis, laboured breathing and wheezing, which subsided eight hours after injection with the compound. Only one mouse in the experimental group injected with 1000 mg/kg MG-192 showed signs of overt toxicity, that is, difficulty breathing and paralysis but these subsided after 30 minutes.

Effect of the test compounds on weight and packed cell volume (PCV) parameters, in comparison with the baseline values, was analyzed using repeated measures analysis of variance (ANOVA).The test compound MG-192 had a significant effect on the body weight of the mice in the group treated with a single injection of 750 mg/kg (p=0.287) and 500 mg/kg (p= 0.026) when compared to the control mice' weight (Table 2). There was a significant difference in the mean PCV of mice administered with 750 mg/kg and 500 mg/kg (p=0.007 and p=0.446) when compared to that of the control mice (Table 2).

Table 2: Effect a single dose administration of MG-192 on mice' weight and PCV.

	Body Weight (g)		PCV (%)	
Drug, dose	Mean difference	p - value	Mean difference	p - value
MG-192 750 mg/kg	-0.75 (±1.399)	0.287	-0.75 (±3.685)	0.007
MG-192 500 mg/kg	1.38 (±1.679)	0.026	-1.33 (±1.633)	0.446
MG-192 100 mg/kg	2.55 (±1.565)	< 0.001	2.60 (±2.434)	0.004
Control (PSG soln)	2.13 (±0.627)	-	0.25 (±3.126)	-

4. Discussion

In 2012, approximately 8000 new cases of human African trypanosomiasis (HAT) infections were reported [14]. Development of new chemotherapeutic agents is necessary if the disease is to be eliminated from the human (and animal) population. With this view, an attempt to apply the structure-activity relationship theory in practice was made, by investigating the biological activity of the test compound MG-192 against trypanosomes, which has similar structural properties to pentamidine. The in vitro experiments done in this study show that the test compound (MG-192) has inhibitory activity against both T. b. rhodesiense and T. congolense bloodstream forms. The compound prevented the multiplication and eventually, survival of the parasites. The propagation of T. b. gambiense isolates in mice, even after immunosuppression of the donor mice and passaging the parasites, yielded parasitaemia of less than antilog 5.4. These levels were too low to allow for in vitro cultivation of any of the gambiense isolates used in the experiment. This illuminates the limitation of using mice in the proliferation of Gambiense species of trypanosomes.

Observation of the mice after a single i.p. injection of MG-192 revealed that the test compound has a profound effect on the respiratory and CNS components of the mice, at extremely high doses. This was exemplified by the mice administered with 5000 mg/kg. The NOAEL was determined to be 1000 mg/kg because only mouse in this experimental group of 6 exhibited symptoms of adverse toxicity, which then subsided half an hour after administration. The mouse may have had altered metabolism, resulting in such an intense reaction to the drug,

albeit for a short time period. Removal of the single mouse in the 1000 mg/kg group warranted the exclusion of that particular group from the rest of the experiment. The test to determine the effect of MG-192 on the body weight and PCV of the experimental mice groups showed that the compound resulted in a slight difference in these parameters at the two dose levels lower than the NOAEL. Pharmacokinetic studies on this new compound are to be done to further explore the properties of MG-192 and if these may have resulted in the change in the mice parameters.

Among the greatest limitations encountered during this study was the high cost of purchasing and maintaining mice. The use of immunosuppressed Swiss white mice in proliferating T. b. gambiense did not yield good results and the low parasitaemia in these mice resulted in the exclusion of the Gambiense species from the study. Another limitation is the generalization that all mice are inherently similar and that changes in the body weight and PCV parameters were caused by external factors, such as the introduction of the test compounds into their bodies: A disparity in the mice was seen in the acute toxicity experiment, where one mouse in the experimental group administered with 1000 mg/kg MG-192 showed temporary signs of overt toxicity while the rest appeared normal. Such changes interfere with the interpretation of results.

Pentamidine is a diamidine compound that shows anti-trypanosomal activity against early-stage T. b. gambiense and has some similar activity against T. b. rhodesiense isolates [3]. It, however, is administered intramuscularly to patients for 7 consecutive days and has been shown to have adverse effects in some patients [15]. A compound drug with the ability to clear trypanosome infections of multiple trypanosome species and that is less toxic and easy to administer to patients would be a great stride towards the elimination of African trypanosomiasis. It is in this hope that MG-192 was manufactured.

5. Conclusion

The novel compound MG-192 exhibits anti-trypanosomal activity against bloodstream forms of T. b. rhodesiense and T. congolense, with few significant effects on murine systems. Further toxicological studies, as well as experiments to determine its efficacy in clearing trypanosome infections in mice are needed in order to inform MG-192's full capabilities. As a recommendation, the use of these conventional tools (in vitro and murine studies to assess the safety and efficacy of potential test compounds as chemotherapeutics) should be used more often in drug development, as they give a more concise indication of the compound's bioactivity. These methods should be used hand-in-hand with computational/predictive methods.

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Reference

[1]. P.P. Simarro, G. Cecchi, J.R. Franco et al. (2012, Oct). "Estimating and Mapping the Population at risk of Sleeping Sickness". PLoS Negl Trop Dis. [Online]. 6(10):e1859. Available:

http://journals.plos.org/plosntds/article?id=10.1371/journal.pntd.0001859. [September 26, 2015].

[2]. G. Zeleke. (2011, Jul.). "Preliminary survey on tsetse flies and trypanosomosis at grazing fields and villages in and around the Nech Sar National Park, Southern Ethiopia". Ethiop Vet J. 15(1), pp.59-67.

[3]. C. Bacchi (2009, April). "Chemotherapy of Human African Trypanosomiasis". Interdiscip Perspect Infect Dis. [Online]. 009(195040). Available: www.hindawi.com/journals/ipid/2009/195040 [December 19, 2015].

[4]. P. Babokhov, A. Sanaolu, W. Oyibo et al. (2013, Jul). "A current analysis of chemotherapy strategies for the treatment of human African trypanosomiasis". [Online]. Pathog Glob Health. 107(5), pp.242–252. Available: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4001453/ [January 9, 2016].

[5]. J.D. Mckinney, A. Richard, C. Waller et al. (2000, Jul.). "The Practice of Structural Activity Relationships (SAR) in Toxicology". Toxicol Sci. [Online]. 56(1). pp. 8-17. Available: http://m.toxsci.oxfordjournals.org/content/56/1/8.long?view=long&pmid=10869449 [January 9, 2016].

[6]. E. Fink and H. Schmidt. "Preclinical testing of trypanocidal drugs in primates: preliminary investigation of an experimental diamidine in vervets" in Recent developments in Medical Research in East Africa. Njogu AR, Tukei PM, Roberts JMD. (Eds), KETRI/KEMRI Nairobi. 1980. pp. 173-182.

[7]. C.J. Bacchi, H.C. Nathan, N. Yarlett et al. (1994, Mar.). "Combination Chemotherapy of drug-resistant Trypanosoma brucei rhodesiense infections in mice using DL-α-diflouromethylornithine and standard trypanocides." Antimicrob Agents Chemother [Online]. 38(3), pp. 563-569. [December 8, 2015].

[8]. J.M. Kagira and N.W. Maina. "Occurrence of multiple drug resistance in T. brucei rhodesiense isolated from sleeping sickness patients." Onderstepoort J Vet Res. vol. 74, pp.17-22, Mar. 2007.

[9]. M.A. Gray and A.S. Peregrine. "An in vitro assay for drug sensitivity of Trypanosoma congolense using in vitro-derived metacyclic trypanosomes." Acta Tropica, vol. 54, pp. 291-300. Sep. 1993.

[10]. N. Maina, J.M. Kagira, P. Maser et al. "Genotypic and phenotypic characterization of Trypanosoma brucei gambiense isolates from Ibba, South Sudan, an area of high melarsoprol treatment failure rate." Acta Trop, vol. 104, pp. 84-90, Nov-Dec. 2007.

[11]. W.J. Herbert and W.H.R. Lumsden. "Trypanosoma brucei: A rapid 'matching' method for estimating the host parasitaemia." Expt. Parasitol. vol. 40, pp. 427-431. Dec. 1976.

[12]. T. Baltz, D. Baltz, C.H. Giroud et al. "Cultivation in a semi-defined medium of animal infective forms of Trypanosoma brucei, T. equiperdum, T. evansi, T. rhodesiense and T. gambiense." EMBO J. vol. 4, pp. 1273-1277. May. 1985.

[13]. M.C. Eisler, J. Brandt, B. Bauer et al. "Standardised tests in mice and cattle for the detection of drug resistance in tsetse-transmitted trypanosomes of African domestic cattle." Vet Parasitol. vol. 97, pp. 171-182. Jun. 2001.

[14]. J. Naessens, H. Kitani, Y. Nakamura et al. (2005, Mar.). "TNF-α mediates the development of anaemia in a murine Trypanosoma brucei rhodesiense infection, but not the anaemia associated with a murine Trypanosoma congolense infection." Clin Exp Immunol. [Online]. 139(3), pp. 405–410. Available: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1809320/ [Oct 13, 2015].

[15]. World Health Organization website. http://www.who.int/trypanosomiasis_african/diagnosis/en/. [Nov. 18, 2014].

[16]. R. Brun, R. Don, RT Jacobs et al. (2011, Jun.). "Development of novel drugs for human African

trypanosomiasis". Future microbial. [Online]. 6(6), pp. 677-691. Available: http://www.futuremedicine.com/doi/full/10.2217/fmb.11.44 [January 18, 2016].