	Report *R # XTN025384*	#	R#XTN025384
		#	INTR#2019-06-21 LC-03456
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		Date:	June 21, 2019

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Efficacy *in vivo* onychomycosis (*Trichophyton mentagrophytes*) study

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List of Abbreviations

IV	intravenous
IP	intraperitoneal
Subq	subcutaneous
mg/kg	milligram per kilogram
ng/mL	nanogram per milliliter
DNA	deoxyribonucleic acid
D5W	5% dextrose in water (278 mmol/L dextrose)
LPX	lipoplex
GLP	Good Laboratory Practice
g	gram
min	minute
hr	hour
LPX	lipoplex
LC	lethal concentration
LD	lethal dose
LC50	concentration causing death in 50%
ppm	parts per million parts
MTD	maximum tolerated dose
OSHA	Occupational Safety and Health Administration
CFR	Code of Federal Regulations

1. STUDY OBJECTIVE

The objective of this non-GLP study is to evaluate preclinically the *in vivo* therapeutic efficacy of topical application of compound of interest (GX-03 / 0.5% PHMB) in animal model of *Trichophyton mentagrophytes* as described in the article Establishment of a Novel Model of Onychomycosis in Rabbits for Evaluation of Antifungal Agents, *ANTIMICROBIAL AGENTS AND CHEMOTHERAPY*, July 2011, p. 3150–3155 Vol. 55, No. 7 doi:10.1128/AAC.00399-11.

2. STUDY ANIMALS AND IACUC REVIEW

Altogen Labs received IACUC study LC03456 protocol approval on May 21, 2019 (Altogen Labs IACUC protocol number 4-05835). Twenty (20) male New Zealand White Rabbits Crl: KBL (NZW) were purchased from the Charles River Laboratories and went through mandatory 1-week acclimatization per IACUC guidelines. All animal procedures and maintenance were conducted in accordance with the institutional guidelines. Rabbits were housed at Altogen Labs animal facility and carcasses frozen and disposed at the end of the study per institutional guidelines.

3. STUDY DESIGN

The administration of the test articles and the animal numbers in each study group are shown in the following experimental design table.

Table 1. Study Design

Group	N	Treatment	Dosing Route	*T.mentagrophytes* Infection	Schedule
1	2	No-infection Control	-	No	-
2	6	No-treatment Control	-	Yes	-
3	12	Test Compound	*Topical*	Yes	Daily

*Note: N = animal number

Total of 20 animals. Study type: non-GLP.

Thirty (30) day study.

4. MATERIALS AND EQUIPMENT

Animals

Species: Male New Zealand White Rabbit (Strain Code 052 (CR), 571 Oakwood). The New Zealand White rabbit was obtained in 1991 by Charles River Canada (CRC) from Kitayama Labs K.K., Japan

Age: 13-14 weeks old

Total number: 20

Animal supplier: Charles River Laboratories

Rabbit onychomycosis model (fungi in the deep layer of the nail) used to evaluate the efficacy of topical antifungal agent (GX-03 / 0.5% PHMB).

Trichophyton mentagrophytes TIMM2789 were applied to the nails of the hind limbs of rabbits. The nails were taken from the rabbits' feet at 0 and 2 weeks after a 2-week infection. *Trichophyton mentagrophytes* administration was performed as described per article Establishment of a Novel Model of Onychomycosis in Rabbits for Evaluation of Antifungal Agents, *Antimicrobial Agents and Chemotherapy* (2011), 55:3150.

Test article administration: Topical

The animals were housed in individual ventilated cages (single rabbit per cage) under the following conditions:

- Temperature: 22-25ºC
- Humidity: 40-60%
- Light cycle: 12 hours light and 12 hours darkness
- Diet: Standard NZW rabbit diet, dry granule food
- Water: sterile water, autoclaved before using
- Cage identification label: per Altogen Labs IACUC
- Animal identification: per Altogen Labs IACUC
- Acclimatization (adapt housing): 7 days mandatory acclimatization period

Test and reference articles

Test article (GX-03 / 0.5% PHMB) was delivered from Global Health Solutions. All articles received in good condition. Test article is GX-03 (0.5% PHMB) formulation of Hexagen Wound Dressing (original formulation is 95% petrolatum and the other 5% is liquid).

5. STUDY DEVIATIONS

None.

6. EXPERIMENTAL METHODS

Trichophyton mentagrophytes administration

Trichophyton mentagrophytes TIMM2789 were applied to the nails of the hind limbs of rabbits. The nails were taken from the rabbits' feet at 0 and 2 weeks after a 2-week infection. *Trichophyton mentagrophytes* administration was performed as described per article Establishment of a Novel Model of Onychomycosis in Rabbits for Evaluation of Antifungal Agents, *Antimicrobial Agents and Chemotherapy* (2011), 55:3150.

Test article administration

Test article (GX-03 / 0.5% PHMB) was administered as topical application daily. A gauze patch was used to wrap together the nail plates of the first to third toes of the hind paw. The treated toe nails were covered with a finger cot (that contained the first to third toes). All work performed in a Biological Safety Cabinet to prepare for topical application.

Nail clipping and DNA/RNA isolation

Nail clipinngs were processed using Qiagen AllPrep Fungal DNA/RNA Isolation Kit (cat# 47154) following manufacturer recommended protocol.

qPCR analysis

Primers and TaqMan probes (IDT):

GAPDH was amplified as an internal reference to adjust for well-to-well variances in the amount of starting template. The *T.mentagrophytes* values were corrected to the GAPDH values (internal reference).

"% Remaining Expression Levels" were calculated as a percentage of *T.mentagrophytes* expression relative to the negative control (no-treatment group). A value of 100% would represent an untreated sample. Values less than 100% exhibit the percent reduction of the target (*T.mentagrophytes*).

TaqMan Fast Advanced Master Mix (ThermoFisher, #4444556) was used for qPCR analysis (384-well format / standard run mode protocol per manufacturers recommended protocol). Concentration of DNA samples were measured prior to analysis and all templates were normalized to 10 ug/ml concentration.

Reference: Journal of Microbiological Methods, Volume 85, Issue 1, 2011, pages 62-66, by G.J.Wisse et al

Observation and data collection

Animals were checked daily for morbidity and mortality. At the time of routine monitoring, the animals were checked for any adverse health effects of *T.mentagrophytes* infection and test article treatment on normal behavior such as mobility, visual estimation of food and water consumption, body weight gain/loss, eye/hair matting and any other abnormal effects. Death and observed clinical signs were recorded per Altogen Labs IACUC

Handling of animals with body weight loss (BWL) during the study and the humane end points of the animals. Body weight loss during the study.

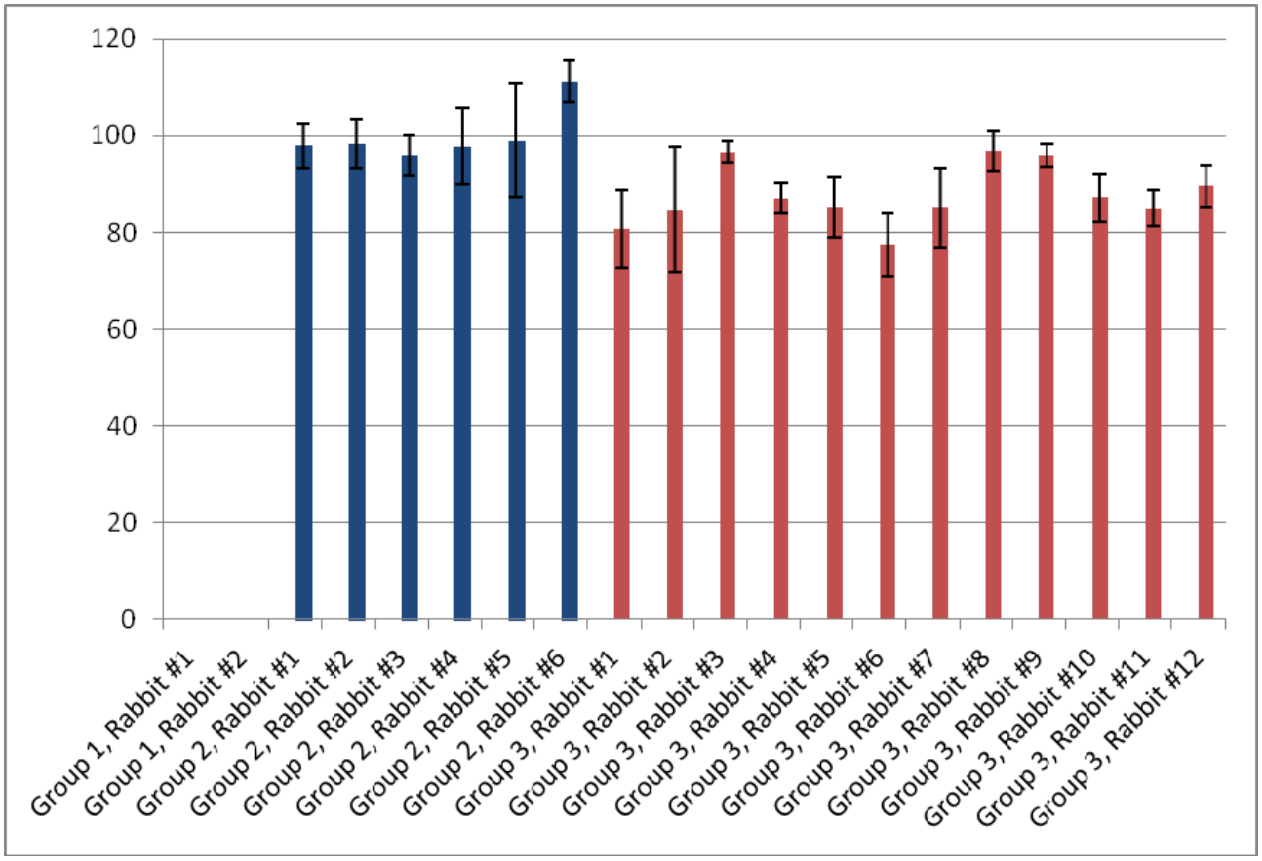
Sacrifice animals when one measurement of body weight loss (BWL)> 20%. Animal research associated with this study was conducted under Altogen Labs IACUC regulatory space. Altogen Labs IACUC guidelines were followed in case of BWL and any other conditions.

7. STATISTICS

Summary statistics, the mean and the standard error of the mean (SEM), are provided for the tumor volume of each group at each time point. Statistical analysis of difference in tumor volume among the groups and the analysis of drug interaction were conducted on the data obtained after the final dose.

8. RESULTS

Figure 1. *T.mentagrophytes* expression of individual animals nail clippings (average value of Group 2 is denoted as 100%). Test article ID and administration schedule indicated in Table 1 above.



*Please see attached Excel file (data.xls) for raw data record.

Figure 2. Raw data and statistical analysis.

Difference	-12.394
Standard error	2.837
95% CI	-18.4074 to -6.3810
t-statistic	-4.369
DF	16
Significance level	P = 0.0005

The differences between the observed means in two independent groups are calculated. A significance value (P-value) and 95% Confidence Interval (CI) of the difference is reported. The P-value is the probability of obtaining the observed difference between the samples if the null hypothesis were true. The null hypothesis is the hypothesis that the difference is 0.

The pooled standard deviation *s*:

$$s = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2}}$$

where s_1 and s_2 are the standard deviations of the two samples with sample sizes n_1 and n_2.

The standard error *se* of the difference between the two means is calculated as:

$$se(\bar{x_1} - \bar{x_2}) = s \times \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}$$

The significance level, or P-value, is calculated using the *t*-test, with the value *t* calculated as:

$$t = \frac{\bar{x_1} - \bar{x_2}}{se(\bar{x_1} - \bar{x_2})}$$

The P-value is the area of the *t* distribution with $n_1 + n_2 - 2$ degrees of freedom, that falls outside $\pm t$

Reference: Altman DG (1991) Practical statistics for medical research. London: Chapman and Hall.

Survival Time

There was no animal death observed within the study.

Clinical Observations

There were no clinical signs or behavioral phenotypes observed within the study (daily cage intensive observation for adverse effect were performed). No animals were observed to have >20% BWL within the study period.

9. ATTACHMENTS

Row data attached in Excel file data format (data.xls)