JPP
Journal of
Pharmacy and Pharmacology

JPP 2010, 62: 257–262

Received August 17, 2009
Accepted November 20, 2009
DOI 10.1211/jpp/62.02.0014
ISSN 0022-3573

Research Paper

In-vitro and in-vivo antivenin activity of 2-[2-(5,5,8a-trimethyl-2-methylene-decahydro-naphthalen-1-yl)-ethylidene]-succinaldehyde against *Ophiophagus hannah* venom

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Abstract

Objectives *Curcuma zedoaroides* A. Chaveerach & T. Tanee, locally known as Wan-Paya-Ngoo-Tua-Mia, is commonly used in the North-Eastern part of Thailand as a 'snakebite antidote'. The aim of this study was to isolate the active compound from the rhizome of *C. zedoaroides*, to determine its structure and to assess its antagonistic activity *in vitro* and *in vivo* against King cobra venom.

Methods The active compound was obtained from *C. zedoaroides* by extraction with acetone followed by purification using column chromatography; its X-ray structure was determined. Its inhibition of venom lethality was studied *in vitro* in rat phrenic nerve-hemidiaphragms and *in vivo* in mice.

Key findings The acetone extract of the *Curcuma* rhizomes contained a C20 dialdehyde, [2-(5,5,8a-trimethyl-2-methylene-decahydro-naphthalen-1-yl)-ethylidene]-succinaldehyde, as the major component. The isolated curcuma dialdehyde was found active *in vitro* and *in vivo* for antivenin activity against the King cobra venom.

Using isolated rat phrenic nerve-hemidiaphragm preparations, a significant antagonistic effect on the inhibition of neuromuscular transmission was observed *in vitro*. Inhibition on muscle contraction, produced by the 4 μg/ml venom, was reversed by 2–16 μg/ml of *Curcuma* dialdehyde in organ bath preparations over a period of 2 h. Mice intraperitoneally injected with 0.75 mg/kg venom and dialdehyde at 100 mg/kg had a significantly increased survival time. Injection of *Curcuma* dialdehyde (100 mg/kg) 30 min before the subcutaneous injection of the venom resulted in a 100% survival time after 2 h compared with 0% for the control group.

Conclusions The *in vitro* and *in vivo* evaluation confirmed the medicinal use of traditional snake plants against snakebites. The bioactivity is linked to an isolated molecule and not a result of synergistic effects of a mixture. The active compound was isolated and the structure fully elucidated, including its stereochemistry. This dialdehyde is a versatile chemical building block and can be easily obtained from this plant source.

Keywords *Curcuma zedoaroides* rhizome; diterpene; *in vivo–in vitro* activity; King cobra antivenom; *Ophiophagus hannah*

Introduction

The traditional use of plants against the effects of snakebite has been long recognized. For many decades scientific attention has been concentrated on numerous plant natural products that have been found active against various snake venoms.[1] A new plant[2] in the genus *Curcuma* (Zingiberaceae), *Curcuma zedoaroides* A. Chaveerach & T. Tanee, with the local name 'Wan-Paya-Ngoo-Tua-Mia', has repute in the North-Eastern part of Thailand as a traditional 'snakebite antidote'. The rhizome of this plant was claimed by the King Cobra Village (Khon Kaen Province, Thailand) to be orally and topically effective in the treatment of poisonous snakebite, which has been documented on television.[3] The locals apply the ground rhizome directly to the bite and eat usually 50–100 g of the

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chopped rhizome. In some villages an ointment-like paste is topically applied to the entire body in a religious ceremony.

Although the accepted standard therapy for snakebite is the immediate administration of a specific antivenom, anaphylaxis and serum sickness frequently result.[4,5] The justified and proven use of a traditional medicine in the treatment of poisonous snakebite would be beneficial for the local people in Thailand, and everywhere in the tropics, and represents, additionally, an interesting starting point of selecting non-toxic compounds for modern drug discovery.

This traditional snakebite treatment can either be very useful, if it is efficient, or extremely dangerous, if it has no therapeutic foundation. Therefore, the aim of this study was to isolate the active compound from the rhizome of *Currcuma zedoaroides* A. Chaveerach & T. Tanee (Wan-Paya-Ngoo-Tua-Mia), to determine its structure and to assess its antagonistic activity *in vitro* and *in vivo* against King cobra venom.

Materials and Methods

Materials

Lyophilized *Ophiophagus hannah* venom was obtained from the Queen Suavabha Memorial Institute, Bangkok, Thailand. The venom was dissolved in normal saline, portioned and kept at –20°C as stock solution. Rhizomes were purchased from the King Cobra Village, Khon Kaen Province, Thailand.

The chemicals and solvents were purchased from Aldrich (Gillingham, UK) and Lancaster Synthesis (Lancaster, UK). Mass spectra were obtained by atmospheric pressure chemical ionisation (APCI), using a Hewlett-Packard 5989b quadrupole instrument (Vienna, Austria). Both proton and carbon NMR spectra were obtained on a Brucker AC 250 instrument (Follanden, Switzerland), calibrated with the solvent reference peak. Infra-red spectra were plotted from KBr discs on a Mattson 300 FTIR spectrophotometer (Coventry, UK).

Experiments were conducted in male Swiss albino mice, 18–22 g, and Sprague Dawley rats, 200–250 g, obtained from the Animal House, Faculty of Medicine, Khon Kaen University. Each experimental group consisted of 6/10 animals. The treatment procedures, according to current UK legislation, were approved by the bioethics committee, Faculty of Medicine, Khon Kaen University (HO 2434-76). Animals had free access to fresh water and food pellets and were exposed to automated 12-h light–dark cycles.

Isolation of the C20-*Curcuma* dialdehyde, 2-[2-(5,5,8a-trimethyl-2-methylene-decahydro-naphthalen-1-yl)-ethylidene]-succinaldehyde

Rhizome (70 g), cut into little slices, was extracted with 350 ml acetone and refluxed for 2 h. This was filtered and the solvent was evaporated off in vacuum to give 6.8 g of the crude aldehyde (9.5%). The crude dialdehyde of 80% purity, based on NMR analysis, was purified by column chromatography with ether–PE (1 : 1) twice. Thirty-five per cent of the pure aldehyde 2-[2-(5,5,8a-trimethyl-2-methylene-decahydro-naphthalen-1-yl)-ethylidene]-succinaldehyde (**CD**) was obtained as it decomposed on the column.

APCI(+): 317 (+OH), 331. FT-IR: ($CHCl_3$) 3451, 3087, 2938, 1726, 1682, 1637, 1386, 1163. ^{1}H NMR: ($CDCl_3$) 9.7+9.5 (1H, 1H, CHO), 6.75 (1H, t, C12H), 4.90+4.40 (1H,1H, s, C17H), 3.40 (2H, d, C14H), 2.40 (3H, m, C11H, C6H), 2.0-1.0 (10H, m), 0.9+0.8+0.7 (9H, s, 3 × Me); ^{13}C NMR: 196.38, 192.60↓ CHO, C15+C16; 146.96, 133.78↑C8, C13, 159.05↓C12, 106.33↑ C17, 55.20, 54.18↓ C6, C7, 32.22, 39.34↑ C1, C5, 34.15, 23.83,14.23↓ Me, 40.54, 38.30, 38.15↑ 36.78, 23.55, 23.52↑ 18.23↑ C14, C11, C9, C10, C2, C3, C4.

Reduction of *Curcuma* dialdehyde to diol

To 2 g of sodium borohydride was added 5 g of the crude dialdehyde **CD** in 100 ml methanol and the mixture was heated under reflux for 20 min. One-hundred millilitres of water was added and the mixture was heated for another 10 min under reflux. Methanol was evaporated off overnight and the remaining solution was extracted three times with 150 ml of dichloromethane (DCM). It was dried with Na_2SO_4 and evaporated off to give 2-[2-(5,5,8a-trimethyl-2methylenen-decahydro-naphthalen-1-yl)-ethyliden]-butane-1,4-diol (**2**) as a white solid. Further purification was achieved by column chromatography with ether–methanol (95 : 5).

APCI(+): 317. FT-IR (KBr): 3246, 2938, 2853, 2369, 1650, 1475, 1463, 1266, 1046, 880. ^{1}H NMR: 5.30 (1H, t, C12H), 4.65+4.30 (2H, s, C17H), 3.70 (2H, s, C16H), 3.50 (2H, t, C15H), 2.65 (2H, bs, OH), 2.25 (2H, m, C14H), 2.0-0.9 (12H, m), 0.8+0.7+0.55 (9H, s, 3 × Me); ^{13}C-NMR ($CDCl_3$)133.23↓ C12, 148.26, 135.33↑ C8, C13; 58.11, 55.31↓ C6, C7; 107.63↑ C17; 33.55, 22.11, 15.11↓ Me; 69.10, 62.82↑ C15, C16; 40.31, 33.62↑ C1, C5; 41.65, 38.74, 36.95, 33.76, 25.54, 22.86, 18.90↑ C14, C11, C1, C2, C3, C4, C9, C10.

X-ray structure of 2-[2-(5,5,8a-trimethyl-2methylenen-decahydro-naphthalen-1-yl)-ethyliden]-butane-1,4-diol

Two independent molecules (Figure 3) occupy the unit cell in space group P1. However, different positions for the hydrogen atoms in the two hydroxymethyl (C19-O1, C39-O21) and the two hydroxyethyl (C17-C18-O2, C37-C38-O22) groups led to major differences in the hydrogen bonding scheme. The sequence of intermolecular hydrogen bonds O1-H1…O2-H2…O22-H22…O21-H21…O1 generated an infinite C(8) zigzag chain. The low overall R-factor (R_{obs} = 0.038) and flat difference electron density map (maximum peak on a difference electron density map 0.12 e Å^{-3}) supported the correctness of their positions.

Preparation of rat phrenic nerve-hemidiaphragms to evaluate *in vitro* the inhibition of venom lethality

Rat phrenic nerve-hemidiaphragms were prepared according to a method developed by the Department of Pharmacology, University of Edinburgh[6] and the contractile responses were studied. The entire nerve–muscle preparation was submerged in 50 ml Kreb's solution with carbogen and the temperature was maintained at 37°C. The phrenic nerve was stimulated with a rectangular-wave pulse of 0.5 ms/0.5 Hz through a bipolar platinum electrode using a Grass Model S-48 stimulator. Muscle contraction was recorded with a force

transducer and Grass Polygraph recorder. The mixed bath solutions (**CD** in dimethyl sulfoxide (DMSO), water, venom) were preincubated in a circulating water bath at 37°C for 30 min and then transferred into an organ bath. Two phrenic nerve-hemidiaphragm preparations were obtained from one rat. One was used as the control with venom only, the second preparation was used to evaluate the venom–antidote combinations at four concentrations in duplicate.

Inhibition of lethality *in vivo*

Mice were injected intraperitoneally with **CD** in DMSO and normal saline and venom subcutaneously at 0.1 ml per mouse; 6/10 mice were used in each treatment group. The survival rates of the mice were recorded every 10 min for 3 h. The experiment was repeated with 10 mice and the survival rate was determined after 24 h.

Statistical methods

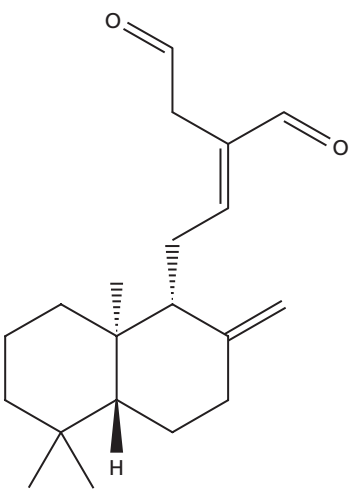
The data were expressed as mean ± SD. One-way analysis of variance and supplementary Tukey's test for pairwise comparison were tested to determine for any significant difference at $P < 0.05$. In-vivo data were analysed by Chi squared test.

Results

Structure

The C20-dialdehyde **CD** was isolated by simple column chromatography from the acetone extract of a new species of *Curcuma zedoaroides*. The chemical structure of the active and major ingredient is outlined in Figure 1. It resembled a labdane template and belongs to the diverse class of diterpenes.

The methyl group (5a position) and the succinaldehyde side chain (1-position) are located below the trans-decaline system. The ring system and the formyl group are orientated in trans position (E-17-ethyliden-labd-12-ene15,16-dial).



CD

Figure 1 *trans-Curcuma* C20-dialdehyde **CD**; 2-[2-(5,5,8a-trimethyl-2-methylene-decahydro-naphthalen-1-yl)-ethylidene]-succinaldehyde; (E)-17-ethyliden-labd-12-ene-15,16-dial

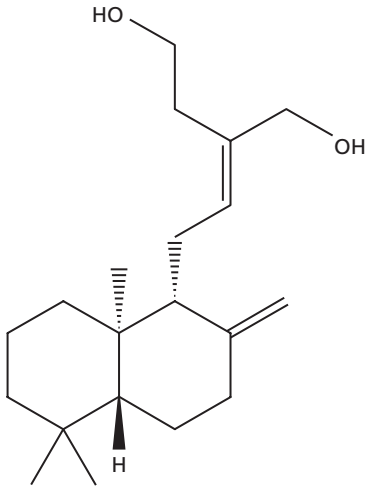


Figure 2 *trans-Curcuma* C20-diol **2**; 2-[2-(5,5,8a-trimethyl-2methylenen-decahydro-naphthalen-1-yl)-ethyliden]-butane-1,4-diol

Attempts were made to modify the sticky dialdehyde **CD**. Reduction of **CD** into the diol **2** provided a white crystalline derivative in a high chemical yield (Figure 2).

Subsequent evaluation of diol **2** showed that the antivenom activity was lost but the compound furnished crystals suitable for X-ray analysis. The crystal structure of diol **2** is outlined in Figure 3.

It was assumed, that the stereochemistry of dialdehyde **CD** and diol **2** are identical and did not change during the reduction.

Inhibition of lethality *in vitro*

The effect of the crude venom and the (E)-17-ethyliden-labd-12-ene-15,16-dial **CD** on the neuromuscular transmission of the rat phrenic nerve-hemidiaphragm are shown in Figure 4. In the control group, 4 μg/ml venom alone gradually and completely inhibited the indirectly-evoked twitches within 30 min. After 10 min, 4 μg/ml venom and 2 μg/ml **CD** resulted in a muscle contraction significantly different from the control. The antagonistic antivenomous effects of the curcuma dialdehyde **CD** increased dose dependently up to 32 μg/ml.

Crude cobra venom and *Curcuma* dialdehyde **CD**, preincubated at 37°C for 1 h in 1 ml volume, showed a total loss of venomous activity, in other words a complete neutralization of the venomous activity (50 ml bath, data not shown, 2 μg/ml × 50 = 100 μg/ml, equivalent to 0.01%).

The 50-times higher incubation concentration simulated the immediate topical application of the ground plant, bearing in mind that the plant juice of the ground material not only contained 0.01%, but nearly 10% of the active dialdehyde.

Inhibition of lethality *in vivo*

When the venom was intraperitoneally injected into mice at a dose of 0.75 mg/kg, all mice died, with a mean survival time of 51 ± 5.48 min. Intraperitoneal injection with (E)-17-ethyliden-labd-12-ene-15,16-dial **CD** at 100 mg/kg showed a significant effect on the survival rate. After 70 min a survival rate of > 80% was observed.

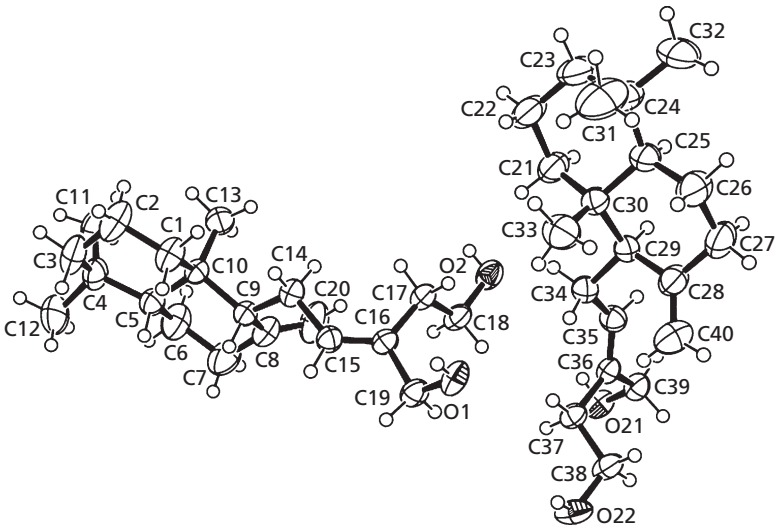


Figure 3 X-ray analysis of the two independent molecules of the *Curcuma* C20-diol **2** with their numbering scheme. Ellipsoids are drawn at the 50% probability level.

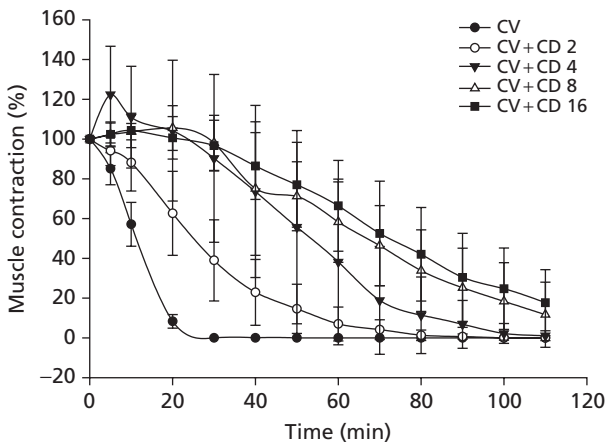


Figure 4 Percentage of muscle contraction of rat phrenic nerve-hemidiaphragms at various times after crude cobra venom (4 μg/ml) alone and venom (4 μg/ml)–*Curcuma* dialdehyde **CD** at concentrations of 2–16 μg/ml. CV, cobra venom.

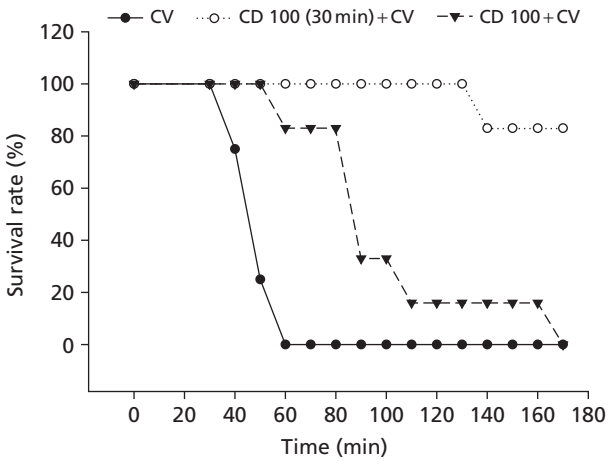


Figure 5 Survival rate of mice at various times after administration of a lethal subcutaneous dose (0.75 mg/kg) of crude cobra venom and a single intraperitoneal dose of 100 mg/kg *Curcuma* dialdehyde **CD** together and 30 min before envenomation. CV, cobra venom.

Pretreatment intraperitoneally with a dose of 100 mg/kg dialdehyde **CD**, 30 min before the lethal injection of the venom subcutaneously, greatly increased the survival time. Mice pretreated with 100 mg/kg **CD** appeared normal for 140 min and more than 80% survived the observation period of 3 h. This is significant ($\chi^2 = 8.6 > 3.8$ at a 5% probability level) and clinically relevant (Figure 5). In a subsequent experiment with 10 mice 50% survived and were found healthy throughout the observation period of 24 h ($n = 10, \chi^2 = 4.3$). For comparison, the Fisher exact test gave a 3.2% probability level, so that even these finding are statistically relevant.

These in-vivo findings correlate well with the in-vitro data, in which a similar concentration range (100 μg/ml ≡ 100 mg/kg) was determined for this lipophilic diterpene-dialdehyde.

Discussion

2-[2-(5,5,8a-Trimethyl-2-methylene-decahydro-naphthalen-1-yl)-ethylidene]-succinaldehyde was isolated as a trace from the rhizomes of *Curcuma longa* L.[7] From this novel *Curcuma* species the new 1R,8aR-trans-dialdehyde **CD** was isolated as the main compound. During the biosynthesis from geranyl diphosphate to labdadienyldiphosphate both stereo-isomers could be generated and examples of both isomers are listed in the Merck index. In RR labdanes, both the methyl group and side chain appear below the decaline system, which is found in andrographolite. In SS labdanes, they are orientated above the plane, as in (−)-sclareol.

The dialdehyde **CD** was reduced with sodium borohydride to the diol **2**. In terms of stereochemistry, the 5-methyl

group and the E-configured side chain are located on the same side, below the trans-decaline ring system. The isomer of RR-diol **2** with opposite stereochemistry was previously reported to display anticancer activity against murine lymphocytic leukaemia P388 (50% inhibitory concentration (IC50) = 6.3 μg/ml), and human gastric adenocarcinoma (IC50 = 8.6 μg/ml) cell lines *in vitro* and was synthesized in nine steps from (−)-sclareol.[8]

Afromodial, a well known labdane-dial with varied bioactivity (e.g. antifungal activity), contained an epoxide ring instead of the methylene group in the 2-position and represented another example of these fascinating biologically active labdane diterpenes.

From this study, (E)-17-ethyliden-labd-12-ene-15,16-dial was found to antagonize the inhibitory action of King cobra venom at the neuromuscular junction, protecting mice from the lethal effects of the crude venom.

Many plant extracts have been reported to possess antagonistic effects against snake venoms[1,9–14] and they mainly worked when preincubated. It is believed that a guest–host complex of the small organic molecule is formed with the venom, but mainly at concentrations that can never be reached *in vitro* or *in vivo*. Here, the labdane dial **CD** worked *in vitro* even at 2 μg/ml and provided excellent protection at a concentration of 40 μg/ml. The in-vitro effect translated into an efficient dose of 100 mg/kg *in vivo* in mice.

The key factor for an efficient therapeutic outcome is the control of pharmacokinetics, thus building up and maintaining a potent concentration of the antidote over time, which then forms an inactive complex with the venomous peptide.

Curcuma longa and other *Curcuma* species are widely used plants in the treatment of snakebite poisoning.[15–17] Cherdchu and colleagues reported the antagonistic effect of the *Curcuma* species named 'Wan-Ngoo' against cobra venom, although it can only be speculated, that the *Curcuma* species studied by Cherdchu contained the *Curcuma* aldehyde **CD**. Most important is the finding that the bioactivity is linked with an active molecule and not a result of synergistic effects of a variety of ingredients.

The 'curcuminoids', visible as yellow dyes, which are present in *Curcuma longa*, were suggested to be the active principles in many *Curcuma* species.[1] Curcumin, one of the curcuminoids, also interacted strongly with biological macromolecules, like serum proteins, albumin and hyaluronic acid.[18] The antivenin effect of *Curcuma longa,* reported by Ferreira *et al.*[17] against the haemorrhagic activity of *Bothrops jararaca,* was due to ar-turmerone.

It is well established that the primary cause of death from cobra envenomation is peripheral respiratory failure due to skeletal neuromuscular blockade.[19] In our study, isolated curcuma aldehyde **CD** antagonized the toxic effect of the cobra venom *in vitro* and *in vivo*. Simple succindialdehyde inactivated the venom at 50-times higher concentrations *in vitro*. Therefore it is assumed that the terpene moiety acted as a template for molecular recognition, guiding the dialdehyde irreversibly to the peptide target (venom). The formed complex is then unable to block the acetylcholine receptors.

Accordingly, the immediate application of chewed *C. zedoaria* rhizome at sites of snakebites, as done by the villagers, could neutralize most of the venom not yet absorbed. The high lipophilicity of the dialdehyde **CD**, based on the labdane template, additionally helped with the absorption and facilitated skin penetration at the site of envenomation.

Conclusions

The in-vitro and in-vivo evaluation confirmed the medicinal use of traditional snake plants against snakebites. All traditional uses, the immediate topical application on the skin and the oral application, are justified.

The bioactivity is linked to an isolated molecule and not a result of synergistic effects of a mixture.

The active compound was isolated and the structure fully elucidated, including its stereochemistry, using X-ray analysis.

The dialdehyde is a versatile building block and can be easily obtained from this plant source. The preparation of various analogues is ongoing; these may have improved stability and a higher potency.

Testing of the active compound against other peptide-based toxins from insects, marine organisms and fungi may be fruitful and may lead to novel medicinal applications.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

The study was partly supported by Fulcrum Television and the University of Khon Kaen, Faculty of Medicine.

Acknowledgement

We are thankful to Dr Mike Davies for excellent technical assistance.

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