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(57) Abstract: The present disclosure relates to a polynucleotide sequence coding for a protein of Trypanosoma species. The disclosure further provides processes for inhibiting said protein for treatment of protozoan infections, Malaria and Surra, by Geldanamycin and 17-AAG. The disclosure also relates to processes for identification of these inhibitions, compositions and method of treatments of said infections. Lastly, the present disclosure also relates to examining the efficacy of Hsp90 inhibitor as an anti-malarial and anti-surra agents.



## POLYNUCLEOTIDE SEQUENCE, PROCESSES, COMPOSITION AND METHODS THEREOF

### TECHNICAL FIELD

5 The present disclosure relates to a polynucleotide sequence coding for a protein of Trypanosoma species. The disclosure further provides processes for inhibiting said protein for treatment of protozoan infections, Malaria and Surra, by Geldanamycin and 17-AAG. The disclosure also relates to processes for identification of these inhibitions, compositions and method of treatments of said infections. Lastly, the present disclosure also relates to  
10 examining the efficacy of Hsp90 inhibitor as an anti-malarial and anti-surra agent.

### BACKGROUND AND PRIOR ART OF THE DISCLOSURE

Malaria is a global health problem and is responsible for about one million deaths annually. *Plasmodium falciparum* is the most deadly of the four species which causes  
15 malaria and is responsible for higher mortality. This problem is aggravated by the emergence of drug resistant species of the parasite. Therefore, it is essential to identify new drug targets in malaria. Currently the drugs available for the treatment of malaria belongs to small number of chemically related compound namely 4 aminoquinolines which includes chloroquine, quinine, mefloquine, amodiaquine and halofantrine; 8  
20 aminoquinolines (primaquine); antifolate which includes pyrimethamine, proguanil, chlorocycloguanil, dapsone and sulphadoxine; artemisinin and its derivatives (artemisinin, artesunate, artemether, arteether, dihydroartemisinin) and hydroxynaphthoquinone atovaquone. Lack of structural diversity in the currently used anti-malarials, leads to development of cross- resistance and emergence of drug resistance stains of parasites.  
25 Additinally, some of the existing drugs have side effects and toxicity issues. Therefore, it is essential to identify new drug targets in malaria.

Previously *Plasmodium falciparum* Hsp90 as a potential drug target and its inhibitors as candidate drugs against malaria has been implicated (Banumathy et al., 2009).  
30 Geldanamycin is a naturally- occurring compound produced by *Streptomyces hygroscopicus* var. *geldanu* (Sasaki et al., 1979). Geldanamycin and its analogs such as

17AAG (17-allylamino-17-demethoxygeldanamycin), 17DMAG (17-dimethylamino-ethylamino-17-demethoxygeldanamycin) have been studied extensively as an anti-cancer agent. Moreover, 17AAG has already completed phase III clinical trial and will soon be available in the market as an anti-cancer drug. Although, the effectiveness of 17AAG in cancer *in vivo* model is well established nothing is known about its efficacy in malaria model.

In cancer cells, Hsp90 inhibition leads to disruption of Hsp90 interaction with its client proteins, which are responsible for the hallmarks of cancer. The exposure of 17AAG leads to degradation of estrogen receptor, serine threonine kinase Raf-1 and Akt in breast cancer, androgen receptor and Her 2 in prostate cancer (Solit et al., 2002), Bcr- Abl and c-Raf1 in leukemia, Erk1/2 and Raf1 in colon adenocarcinoma cells and erbB1 and erbB2 in non-small cell lung carcinoma (NSCLC), all of which are Hsp90 clients.

Further, several efforts have been made in the field of cancer to evaluate the effectiveness of 17AAG as an anti-cancer agent in mouse cancer model as well as human clinical trial. A toxicology study of 17AAG in non-tumor bearing mice have shown that treatment with three consecutive 5 day cycles of 75 mg/kg or more causes toxicity as evident by the weight loss, elevated liver transaminase levels, anemia and death however a less frequent dosing up to 150 mg/kg/day of drug is safe (Solit et al., 2002). A schedule comprising of three consecutive weekly 5-day cycles, at 50 mg/kg 17AAG caused 80% growth inhibition of CWRSA6 tumor growth (Solit et al., 2002). Newcomb et al., showed that the administration of 17AAG (50mg/kg) intraperitoneally three times a week for up to 28 days exhibits anti-tumor effect on GL261 intracranial tumors. A study in multiple myeloma SCID/NOD model showed that a 50mg/ kg intraperitoneal injection of 17AAG for 5 consecutive days followed by 2 days off therapy in each cycle, for total of 4 cycles prolonged median survival of mice (Mitsiades et al., 2006). Interestingly, intraperitoneal injection of 17AAG showed a bioavailability of 100%, far higher as compared to when given orally (Egorin et al., 2001). Plasma pharmacokinetics study of 17AAG in mice model showed that an intravenous dose of 40 mg/ kg produced a peak plasma

concentration of 20.2-38.4  $\mu\text{g/ml}$  after 5 min of injection while intraperitoneal injection produced a peak plasma concentration of 11  $\mu\text{g/ml}$  after 60 min of injection. In both of the cases plasma concentration declined to less than lower limit of quantitation after 240 min of injection. 17AAG was found to be metabolized to 17AG and other metabolites after injection. Increasing doses of 17AAG resulted in higher plasma concentrations and exposure to 17AAG. 17AAG was found to be widely distributed to tissue, highest in lung followed by liver, spleen, heart, kidney, brain and skeletal muscle. At any time the concentration of 17AAG in tissue was found to more than its plasma concentration. 17AAG was detected in almost all tissue for at least 8 hr and in some for even after 24 hr of drug delivery (Egorin et al., 2001).

After the success in the preclinical model for various tumors, 17AAG has been evaluated for its effectiveness as an anti-cancer agent in human clinical trials. A phase I trial in patient with refractory advanced cancer showed that 17AAG doses between 10 and 295  $\text{mg/m}^2$  is well tolerated and also 17AAG showed liner pharmacokinetics. Further, a dose of 295  $\text{mg/m}^2$  weekly x 3, repeated every 4 weeks was recommended for future study (Ramanathan et al., 2005). Another phase I trial of 17AAG in patients with advanced cancer indicated a maximum tolerated dose (MTD) of 220  $\text{mg/m}^2$  for twice-weekly schedule. This study also showed that at MTD the drug affects the target in normal tissue. Phase I pharmacokinetics and pharmacodynamic study of 17AAG in patient with advanced malignancies indicated that at 450  $\text{mg/m}^2/\text{week}$ , 17AAG plasma concentration was > 120  $\text{nmol/L}$  which was similar to the mean  $\text{IC}_{50}$  of 17 AAG across NCI 60 tumor cell line panel and most importantly this was maintained for 24hr. However, out of 30 studied patient, three showed 3 grade diarrhea (one at 320  $\text{mg/m}^2/\text{week}$  and two at 450  $\text{mg/m}^2/\text{week}$ ) and one showed 3 to 4 hepatotoxicity at 450  $\text{mg/kg/week}$ . But due to its tolerated toxicity profile and ability to inhibit target in patients, this dose was recommended for further study (Banerji et al., 2005). As an outcome of several such studies, Cancer Therapy Evaluation Program (CTEP) of the National Cancer Institute recommended a phase II dose/schedule regimes of 220  $\text{mg/m}^2$  (mg per square meter of body surface area (BSA) of the patient or subject) administered twice weekly for 2 out of 3

weeks, 450 mg/m<sup>2</sup> administered once a week continuously or with a rest or break, and 300 mg/m<sup>2</sup> once a week for 3 weeks out of 4 weeks for further study. Despite the fact that 17AAG already entered phase III clinical trial as an anti cancer agent (Usmani et al., 2009), 17AAG has still not been approved by any authority for use in treatment of any cancer or for that matter any other disease.

#### STATEMENT OF DISCLOSURE

Accordingly the present disclosure relates to a polynucleotide sequence set forth as SEQ ID NO: 1; a process for inhibiting Heat Shock Protein 90 (HSP90) of Trypanosoma species or Plasmodium species by 17-allylamino-17-demethoxygeldanamycin (17-AAG), optionally along with at least one pharmaceutically acceptable excipient, said process comprising act of contacting said 17-AAG with the HSP90; a process for inhibiting Heat Shock Protein 90 (HSP90) of Trypanosoma species by Geldanamycin, optionally along with at least one pharmaceutically acceptable excipient, said process comprising act of contacting said Geldanamycin with the HSP90; a process for identifying inhibition of Heat Shock Protein 90 of Trypanosoma species by compounds selected from a group comprising Geldanamycin (GA) and 17-allylamino-17-demethoxygeldanamycin (17-AAG) optionally along with at least one pharmaceutically acceptable excipient, said process comprising acts of- a) isolating and amplifying sequence coding for the *Trypanosoma evansi* HSP 90 (TeHSP90) to obtain an amplicon, b) cloning the amplicon into a vector to obtain recombinant TeHSP90 sequence and c) infecting subject with the recombinant TeHSP90 sequence followed by administering GA or 17AAG to identify said Trypanosoma HSP90 inhibition; a process for identifying inhibition of Heat Shock Protein 90 of Plasmodium species by 17-allylamino-17-demethoxygeldanamycin (17-AAG), optionally along with at least one pharmaceutically acceptable excipient, said process comprising acts of- a) isolating and amplifying sequence coding for the *Plasmodium berghei* HSP 90 (PbHSP90) to obtain an amplicon, b) cloning the amplicon into a vector to obtain recombinant PbHSP90 sequence and c) infecting subject with the recombinant PbHSP90 sequence followed by administering 17AAG to identify said Plasmodium HSP90 inhibition; a method of treating Malaria or Surra, said method comprising act of

administering 17-allylamino-17-demethoxygeldanamycin (17-AAG) optionally along with at least one pharmaceutically acceptable excipient, to a subject in need thereof; and a method of treating Surra, said method comprising act of administering Geldanamycin, optionally along with at least one pharmaceutically acceptable excipient, to a subject in need thereof.

## BRIEF DESCRIPTION OF THE ACCOMPANYING FIGURES

**Figure 1:** PfHsp90 exhibits higher ATPase activity.

A) Dissociation constant ( $K_d$ ) determination of ATP using tryptophan fluorescence.

10 Change in fluorescence ( $\Delta F$ ) plotted against increasing ATP concentration.

B) ATP hydrolysis by PfHsp90. Initial rate of ATP hydrolysis is measured by the direct conversion of radiolabeled ATP to ADP. Michaelis-Menten plot showing  $\mu$ mole of ATP hydrolyzed per min against increasing concentrations of ATP ( $\mu$ M).

C) A related rate of ATP hydrolysis of Hsp90, from chicken, human, yeast and  
15 *Plasmodium*. *Plasmodium* Hsp90 has higher ATPase activity as compared to human and chicken and is similar to Yeast Hsp90.

**Figure 2:** Efficacy of a 17AAG in a rodent model of malaria.

A) Represents Giemsa stained tail smear of 17AAG untreated and treated *P. berghei* infected mice. 17AAG untreated mice smear (left side), 17 AAG treated mice (right side)  
20 after six days.

B) Percentage parasitaemia versus days post infection.

C) Percentage viability of 17AAG treated/untreated mice plotted against time.

**Figure 3:** GA immobilized beads specifically pull down TeHsp90 from *T.evansi* lysate. GA pull down fraction for SDS PAGE (A) and two dimensional electrophoresis (B).

25 **Figure 4:** Cloning sequencing and purification of TeHsp90 from isolated *T. evansi* from infected mice.

A) PCR amplicon for TeHp90.

B) TeHsp90 clone is confirmed by the double digestion of clone using restriction enzyme which is introduced in the primer used for cloning in pRSETA. Release of insert of 2 Kb  
30 size from pRSETA confirms the presence of TeHsp90 sequence.

C) SDS-PAGE of purified TeHsp90.

D) Coding sequence for TeHsp90. TeHsp90 sequence is obtained by sequencing of positive clone as described in the section “**Cloning and purification of TeHsp90**”.

E) Sequence alignment of Hsp90 from *T. evansi* (TeHsp90) and *T. brucei* showing 98% of sequence identity.

**Figure 5:** Determination of binding affinity of GA towards TeHsp90.

Change in fluorescence ( $\Delta F$ ) is plotted against GA concentrations for TeHsp90 which gives a dissociation constant of 0.94  $\mu$ M.

**Figure 6:** A) and B) show in vivo efficacy of Hsp90 inhibitors on the survivability of *T. evansi* infection in mice

## DETAILED DESCRIPTION OF THE DISCLOSURE

The present disclosure relates to a polynucleotide sequence set forth as SEQ ID NO: 1.

In an embodiment of the present disclosure the polynucleotide codes for Heat Shock Protein 90 (HSP90) of *Trypanosoma evansi*.

The present disclosure also relates to a process for inhibiting Heat Shock Protein 90 (HSP90) of *Trypanosoma* species or *Plasmodium* species by 17-allylamino-17-demethoxygeldanamycin (17-AAG), optionally along with at least one pharmaceutically acceptable excipient, said process comprising act of contacting said 17-AAG with the HSP90.

The present disclosure also relates to a process for inhibiting Heat Shock Protein 90 (HSP90) of *Trypanosoma* species by Geldanamycin, optionally along with at least one pharmaceutically acceptable excipient, said process comprising act of contacting said Geldanamycin with the HSP90.

In an embodiment of the present disclosure, the HSP90 is obtained from samples infected with said species; and wherein the samples are selected from a group comprising blood, serum, lymph, urine and plasma or any combination thereof.

In another embodiment of the present disclosure, the HSP90 is contacted with the 17-AAG at a concentration ranging from about 20mg/kg to about 60mg/kg of body weight of subject from which the sample is derived.

In yet another embodiment of the present disclosure, the Geldanamycin is at a concentration ranging from about 20  $\mu$ l/mg to about 50  $\mu$ l/mg of lysate of the protein.

The present disclosure also relates to a process for identifying inhibition of Heat Shock Protein 90 of *Trypanosoma* species by compounds selected from a group comprising Geldanamycin (GA) and 17-allylamino-17-demethoxygeldanamycin (17-AAG) optionally along with at least one pharmaceutically acceptable excipient, said process comprising acts of:

- a) isolating and amplifying sequence coding for the *Trypanosoma evansi* HSP 90 (TeHSP90) to obtain an amplicon;
- b) cloning the amplicon into a vector to obtain recombinant TeHSP90 sequence; and
- c) infecting subject with the recombinant TeHSP90 sequence followed by administering GA or 17AAG to identify said *Trypanosoma* HSP90 inhibition.

The present disclosure also relates to a process for identifying inhibition of Heat Shock Protein 90 of *Plasmodium* species by 17-allylamino-17-demethoxygeldanamycin (17-AAG), optionally along with at least one pharmaceutically acceptable excipient, said process comprising acts of:

- a) isolating and amplifying sequence coding for the *Plasmodium berghei* HSP 90 (PbHSP90) to obtain an amplicon;

- b) cloning the amplicon into a vector to obtain recombinant PbHSP90 sequence; and
- c) infecting subject with the recombinant PbHSP90 sequence followed by administering 17AAG to identify said Plasmodium HSP90 inhibition.

5

In an embodiment of the present disclosure, the 17-AAG is administered intraperitoneally in the range of about 20mg/kg to about 60mg/kg of body weight of the subject, wherein the subject is mammal.

- 10 In another embodiment of the present disclosure, the Geldanamycin is at a concentration ranging from about 20  $\mu$ l/mg to about 50  $\mu$ l/mg of lysate of the HSP90.

In yet another embodiment of the present disclosure, survival rate of the subject is enhanced by 60% post administering of the 17-AAG.

15

In still another embodiment of the present disclosure, survival rate of the subject is enhanced by about 30% to about 50% post administering of the 17-AAG.

- 20 In still another embodiment of the present disclosure, the Plasmodium species is *Plasmodium berghei* and the Trypanosoma species is *Trypanosoma evansi*.

In still another embodiment of the present disclosure, the administering is carried out intraperitoneally.

- 25 The present disclosure also relates to a method of treating Malaria or Surra, said method comprising act of administering 17-allylamino-17-demethoxygeldanamycin (17-AAG) optionally along with at least one pharmaceutically acceptable excipient, to a subject in need thereof.

The present disclosure also relates to a method of treating Surra, said method comprising act of administering Geldanamycin, optionally along with at least one pharmaceutically acceptable excipient, to a subject in need thereof.

- 5 In an embodiment of the present disclosure, the administering inhibits Heat Shock Protein 90 (HSP90) of species causing the Malaria and the Surra; and wherein the subject is mammal.

In another embodiment of the present disclosure, the excipient is selected from a group comprising gums, granulating agents, binding agents, lubricating agents, disintegrating  
10 agents, sweetening agents, coloring agents, flavoring agents, coating agents, plasticizers, preservatives, suspending agents, emulsifying agents, antistatic agents, and spheronization agents or any combination thereof.

Malaria is one of the most wide spread diseases. Most of the existing compounds used for  
15 the treatment of malaria belong to a small group of chemically related compounds. Moreover, resistance of parasite to a particular drug leads to the development of cross resistance. As a result parasites are now becoming resistant to almost all drugs available in the market. The only drug, for which no resistance was earlier observed, was artemisinin. However, recent reports have suggested the presence of artemisinin parasites in Cambodia.  
20 Therefore, it is essential to identify new drugs and drug targets in malaria. Additionally, these new drug should belong to a family other than existing ones to avoid any cross-resistance.

The present disclosure discloses that 17-AAG, belongs to benzoquinone ansamycine, a  
25 completely different family from existing drugs, inhibits parasite growth in murine model of malaria. This is directed towards heat shock protein 90 from parasites which shows a lesser tendency towards single nucleotide polymorphisms and therefore, is less likely to develop drug resistant form.

Further, the present disclosure involves the interaction between drug and protein and the method for treating malaria in the pre-clinical model of malaria using Hsp90 inhibitor 17AAG.

#### 5 **PfHsp90 is a Hyperactive ATPase**

Hsp90 from different organisms are known to bind ATP and possess a weak ATPase activity. The present disclosure uses tryptophan fluorescence measurement to demonstrate PfHsp90 binding to ATP and quantitate its binding affinity. In general, ATP-bound and -free forms of Hsp90 exhibit measurable difference in tryptophan fluorescence intensity.

10 Purified PfHsp90 was incubated with different concentrations of ATP and difference in the tryptophan fluorescence was plotted against increasing concentrations of ATP. Dissociation constant was determined by analyzing the data using Graphpad<sup>R</sup> prism 5.0. The dissociation constant ( $K_d$ ) of ATP with PfHsp90 was determined to be  $168 \pm 25$  (Figure 1A). Using the same experimental approach, previous studies found ATP to bind human

15 Hsp90 and yeast Hsp90 with a dissociation constant of  $240 \pm 14$  and  $132 \pm 47$  respectively (Table 1) [McLaughlin et al., 2004; Prodromou et al., 1997 13]. This result suggests that PfHsp90 binds ATP with a 30% higher affinity as compared to its human host.

To examine the ATPase activity of PfHsp90, the purified protein with different

20 concentrations of ( $\gamma$ - $^{32}\text{P}$ \*) ATP is incubated and the direct conversion of ( $\gamma$ - $^{32}\text{P}$ \*) ATP to ( $\gamma$ - $^{32}\text{Pi}$ \*) by thin layer chromatography is monitored. As observed from Figure 1B, the ATPase activity of PfHsp90 follows Michaelis-Menten kinetics. PfHsp90 hydrolysed ATP with a  $K_m$  of  $611 \mu\text{M}$  and  $K_{cat}$  of  $8.1 \times 10^{-2} \text{ m}^{-1}$ . A comparison of the thermodynamic properties of ATP binding among PfHsp90 and hHsp90 revealed that PfHsp90 had higher

25 catalytic efficiency of  $13.3 \times 10^{-5} \text{ m}^{-1} \mu\text{M}^{-1}$  (Figure 1C and Table1) which is three times more than that of its human host [Owen et al., 2002].

**Since the molecule is binding to ATP binding pocket of PfHsp90, due to hyperactive ATPase for PfHsp90, binding of GA and its analogues 17AAG to PfHsp90 increases,**

30 **as the molecule mimics ATP binding to PfHsp90.**

**Table 1. Biochemical properties of Hsp90 from different organisms**

| Organism             | ATP              |                         |                                       |              | Hsp90 ATPase     | GA                 |
|----------------------|------------------|-------------------------|---------------------------------------|--------------|------------------|--------------------|
|                      | Km<br>( $\mu$ M) | Kcat (m <sup>-1</sup> ) | Kcat/ Km<br>(m <sup>-1</sup> $\mu$ M) | Kd( $\mu$ M) | IC <sub>50</sub> | Kd ( $\mu$ M)      |
| <i>P. falciparum</i> | 611              | $9.9 \times 10^{-2}$    | $16.2 \times 10^{-5}$                 | 168 $\pm$ 25 | 207              | 1.05               |
| <i>H. sapiens</i>    | 324              | $1.5 \times 10^{-2}$    | $4.5 \times 10^{-5}$                  | 240 $\pm$ 14 | 702              | 4.4<br>0.480<br>>1 |
| <i>S. cerevisiae</i> | 511              | $8.0 \times 10^{-2}$    | $15.6 \times 10^{-5}$                 | 132 $\pm$ 47 | N.D              | 1.2                |
| <i>G. gallus</i>     | 1530             | $2.5 \times 10^{-2}$    | $1.6 \times 10^{-5}$                  | N.D          | N.D              | N.D                |

- 5 **Km:** Michaelis Menton constant Km is a substrate concentration at which reaction rate is half of Vmax.

**Kcat:** It is a direct measure of the catalytic production of product under optimum conditions also called as turnover number.

**Kd:** Dissociation constant

- 10 N.D-Not determined

The present disclosure is further elaborated and detailed with the help of the examples provided herein. However, the examples and figures furnished herein are presented for the purpose of illustration only and in no way intended to limit the scope of the disclosure.

15

## EXAMPLES

### Example 1:

#### Treatment of Malaria by 17AAG

- 20 **In vivo efficacy of PfHsp90 inhibitors [17AAG] in rodent model of malaria**

Present disclosure examines the ability of 17AAG to inhibit parasite growth in *P. berghei* infected mice. For this, Peter's four days suppressive test against *P. berghei* infection in mice is performed. Female Swiss mice (22–25 g) are infected intraperitoneally with 100  $\mu$ l of *P. berghei* infected blood. The no. of infected RBCs were  $10^6$ . After confirmation of

infection by Giemsa stained tail smears, they are divided into two groups each having four mice. 17AAG is dissolved in 20% Cremophor<sup>R</sup> EL (Sigma) in PBS (50 mg/kg body weight) and injected intraperitoneally for four consecutive days. Vehicle-treated infected mice served as control. Survival of mice is monitored for a period of 3 weeks. Every alternate day tail smears are taken and the number of infected RBC's are counted and plotted against days post infection. Percent survival is plotted as a function of time. The experiment involving mice study has been conducted adhering to the institution's guidelines for animal husbandry at Indian Institute of Science.

Figure 2A is a representative Giemsa stained tail smear of 17AAG untreated and treated mice. As evident from the smear, 17AAG is able to inhibit parasite growth. Figure 2B shows the percentage parasitemia observed in the control and drug treated experimental mice until the 6<sup>th</sup> day following infection, by when majority of control mice had succumbed to infection. It is evident that in the control group the parasitemia rose steadily peaking at 80-90% until death of the animal while in drug treated mice the parasitemia is significantly attenuated resulting in about two-fold prolonged survival time of the drug treated mice (Figure 2B). An overall survival rate of 30-50% in 17AAG treated as compared to 0% in vehicle treated animals is observed 14 days post infection. However, the observation was further carried out for a period beyond the initial 14 days and upto 21 days post infection, wherein the results obtained mimicked the results as shown in Figure 2C. The log rank test 'P' value is found to be 0.00692 ( $P < 0.01$ ). The low 'P' value suggests that the difference as observed by the survivability curve is not merely a chance, but is an outcome of drug treatment. The results shown here provide a proof of principle for the efficacy of an Hsp90 inhibitor 17AAG as an anti-malarial in the pre-clinical rodent model of malaria.

### Example 2:

**Treatment of surra by Geldanamycin and 17-AAG, as Hsp90 inhibitors in *in vivo* mice model**

*Trypanosoma evansi* is a protozoan parasite which causes surra. Surra is one of the major health concerns among the domestic animals like camels, horses, cattles and buffaloes. *T. evansi* causes an acute form of the disease, which when untreated can lead to death of the animal. Further, the emergence of drug resistant parasites against most extensively used drugs such as suramin and quinapyramine is a matter of concern. In addition, unavailability of *T. evansi* genome sequence makes it even more challenging for research community to search for new drug targets. Recent studies have implicated heat shock protein 90 (Hsp90) in the development of many protozoan parasites. However, Hsp90 from different organisms do not always show similar kind of behavior with respect to their interaction as well as inhibition by Hsp90 inhibitors. For example, Hsp90 of *C.elegans*, a free living nematode was unable to bind GA (David et al., 2003), however, Hsp90 of parasitic nematode *Brugia Pahang* binds to GA (Devaney et al., 2005). This suggests that high degree of overall similarity in the sequence does not always ensure similarity in binding to a particular inhibitor. Although it is known that GA can bind to Hsp90 from *Plasmodium*, there is nothing known about its direct interaction with *T. evansi* Hsp90. Additionally, as *T. evansi* genome is not sequenced, there is no information about the sequence of TeHsp90 itself. Therefore, in the present disclosure the characterization of Hsp90 from *T. evansi* in terms of its interaction with Hsp90 inhibitor as well as potential of TeHSP90 as a drug target is systematically carried out. In the present disclosure it is shown that geldanamycin coupled beads specifically pull down *T. evansi* Hsp90 (TeHsp90) from the lysate prepared from purified parasites from *T. evansi* infected mice (Figure 3). In the present disclosure TeHsp90 obtained from parasites in *T. evansi* infected mice is cloned and purified and sequenced using primer walking approach (Figures 4, 5). Finally, it is shown that a four day treatment schedule of 17AAG inhibits *T. evansi* growth in mice. Most importantly, 17AAG is able to cure 60% *T. evansi* infected mice and these mice appear normal till 35 days post infection (Figure 6) (time under observation). However, the observation was further carried out for a period beyond the initial 35 days and upto 90 days post infection, wherein the results obtained mimicked the results as shown in Figure 6B.

#### **Example 2A:**

### **Treatment of Surra by Geldanamycin**

#### **GA-coupled beads specifically pull down TeHsp90.**

GA coupled beads are prepared as previously described by Pavithra et al, 2004. *T. evansi* is purified from infected mice blood using DEAE-cellulose (Roy et al, 2010). Purified *T. evansi* is lysed in a buffer containing 50 mM Tris pH 7.5, 0.1% NP40, 2 mM EDTA, 100 mM NaCl, 1mM sodium orthovanadate and protease inhibitor mix. 3mg protein/parasite lysate was divided into two groups control and tests (1.5 mg each). The parasite lysate is incubated overnight with GA coupled beads at 4°C The reaction was carried out in each group with 50µl of control and test beads (GA coupled beads). . Uncoupled beads serve as a control. As it can be seen from Figure 3, GA- coupled beads specifically pull down a band corresponding to 83 kDa protein, which upon immunoblotting cross-reacts with antibody against *Dictyostelium discoideum* Hsp90. The identity of this band is further confirmed by mass spectrometry which gave first hits for Hsp90 from *T. brucei*, a closely related species of *T. evansi*, whose sequence is known. The above results suggest that GA specifically binds to TeHsp90.

#### **Cloning and purification of TeHsp90.**

TeHsp90 is cloned by preparing cDNA from total RNA isolated from *T. evansi* isolated from infected mice by RT-PCR. TeHsp90 is amplified using the primers based on the presence of conserved MEEVD amino acid present at the C-terminal end of most cytosolic Hsp90 and also using extreme N-terminal sequence and C-terminal sequence identified during MS/MS analysis of GA pull down band. Indeed sequencing of the GA pull down band which is identified as Hsp90 by Mass spectrometry has showed MEEVD sequence at its extreme end. The amplicon is cloned into pRSETA (Figures 4A, 4B). The clone is sequenced using primer walking approach as well as using T7 forward and T7 reverse primer. The present disclosure for the first time provides the sequence for TeHsp90 (Figure 4D).

TeHsp90 shows a 98% sequence identity with closely related species *T. brucei*, from which *T. evansi* is thought to have evolved (Hoare et al., 1972; Lai et al., 2008 ) (Figure

4E). *T. brucei* causes sleeping sickness in humans and nagana in animals in Africa. For purification, the recombinant TeHsp90 is expressed in *E. coli* Rosetta strain and purified using Ni-NTA affinity chromatography (Qiagen) (Figure 4C).

#### 5 **Purified TeHsp90 binds to GA**

The biochemical property of GA binding to purified TeHsp90 is determined thereafter. Purified TeHsp90 is incubated with varying concentrations of GA and the difference in tryptophan fluorescence is plotted against increasing concentrations of GA (Figure 1A). Dissociation constant is obtained by analyzing the resulting data using nonlinear regression  
 10 for single site specific binding using Graphpad<sup>R</sup> prism 5.0. The binding affinity of GA towards TeHsp90 is found to be 0.94 $\mu$ M (Figure 5). Hence, Geldanamycin is shown to successfully inhibit the HSP90 of *T. evansi*.

#### **Example 2B:**

#### **Treatment of Surra by 17-AAG**

#### 15 **GA analogue 17AAG cures *T. evansi* infection in mice.**

The ability of 17AAG in inhibiting *T. evansi* infection at pre-clinical level is further examined. For this, swiss female mice are infected with 10<sup>5</sup> cells of *T. evansi* and treated with 17AAG for four continuous days. 17 AAG is administered intraperitoneally at two different concentrations (1) 30mg/ kg wt and (2) 50 mg / kg wt. *T. evansi* infected mice  
 20 group which are injected intraperitoneally with vehicle (20% cremophore in PBS) only serve as an untreated control. Everyday a drop of blood is collected from each mice and the number of parasites are counted using hematocytometer. Average number of parasites in each group are plotted against days post infection. Figure 6A shows the number of parasites in untreated and 17AAG treated mice groups. It is evident that in control mice the  
 25 number of parasites increases rapidly to 10<sup>8</sup> parasites/ ml and resulted in death of all mice by 9<sup>th</sup> day following infection, while in the drug treated mice no parasites are detected resulting in curing of disease in those mice. An overall survival rate of 60 % is observed for 35 days (Figure 6B) in 17 AAG treated mice and survival was recorded for more than 90 days. Further, the target of 17AAG, in this case TeHs90 is 98 % identical to TbHsp90.

**CONCLUSION**

In the present disclosure Hsp90 inhibitor is shown to be effective in inhibiting the growth of two parasites, *P. berghei* and *T. evansi*, in *in vivo* mice model. The results shown in the present disclosure suggest that Hsp90 inhibitors, such as Geldanamycin and 17-AAG can  
5 be used in the treatment of wide range of disease caused by protozoan parasites both in human and animals.

**We Claim:**

- 1) A polynucleotide sequence set forth as SEQ ID NO: 1.
- 2) The sequence as claimed in claim 1, wherein the polynucleotide codes for Heat Shock Protein 90 (HSP90) of *Trypanosoma evansi*.
- 5 3) A process for inhibiting Heat Shock Protein 90 (HSP90) of Trypanosoma species or Plasmodium species by 17-allylamino-17-demethoxygeldanamycin (17-AAG), optionally along with at least one pharmaceutically acceptable excipient, said process comprising act of contacting said 17-AAG with the HSP90.
- 10 4) A process for inhibiting Heat Shock Protein 90 (HSP90) of Trypanosoma species by Geldanamycin, optionally along with at least one pharmaceutically acceptable excipient, said process comprising act of contacting said Geldanamycin with the HSP90.
- 15 5) The processes as claimed in claims 3 and 4, wherein the HSP90 is obtained from samples infected with said species; and wherein the samples are selected from a group comprising blood, serum, lymph, urine and plasma or any combination thereof.
- 6) The process as claimed in claim 5, wherein the HSP90 is contacted with the 17-AAG at a concentration ranging from about 20mg/kg to about 60mg/kg of body weight of subject from which the sample is derived.
- 20 7) The process as claimed in claim 4, wherein the Geldanamycin is at a concentration ranging from about 20  $\mu$ l/mg to about 50  $\mu$ l/mg of lysate of the protein.
- 8) A process for identifying inhibition of Heat Shock Protein 90 of Trypanosoma species by compounds selected from a group comprising Geldanamycin (GA) and 17-allylamino-17-demethoxygeldanamycin (17-AAG) optionally along with at least one pharmaceutically acceptable excipient, said process comprising acts of :  
25     a) isolating and amplifying sequence coding for the *Trypanosoma evansi* HSP 90 (TeHSP90) to obtain an amplicon;  
      b) cloning the amplicon into a vector to obtain recombinant TeHSP90 sequence; and

- c) infecting subject with the recombinant TeHSP90 sequence followed by administering GA or 17AAG to identify said Trypanosoma HSP90 inhibition.
- 9) A process for identifying inhibition of Heat Shock Protein 90 of Plasmodium species by 17-allylamino-17-demethoxygeldanamycin (17-AAG), optionally along with at least one pharmaceutically acceptable excipient, said process comprising acts of:
- a) isolating and amplifying sequence coding for the *Plasmodium berghei* HSP 90 (PbHSP90) to obtain an amplicon;
- b) cloning the amplicon into a vector to obtain recombinant PbHSP90 sequence; and
- c) infecting subject with the recombinant PbHSP90 sequence followed by administering 17AAG to identify said Plasmodium HSP90 inhibition.
- 10) The processes as claimed in claims 8 and 9, wherein the 17-AAG is administered intraperitoneally in the range of about 20mg/kg to about 60mg/kg of body weight of the subject, wherein the subject is mammal.
- 11) The process as claimed in claim 8, wherein the Geldanamycin is at a concentration ranging from about 20 µl/mg to about 50 µl/mg of lysate of the HSP90.
- 12) The process as claimed in claim 8, wherein survival rate of the subject is enhanced by 60% post administering of the 17-AAG.
- 13) The process as claimed in claim 9, wherein survival rate of the subject is enhanced by about 30% to about 50% post administering of the 17-AAG.
- 14) The processes as claimed in claims 8 and 9, wherein the Plasmodium species is *Plasmodium berghei* and the Trypanosoma species is *Trypanosoma evansi*.
- 15) The processes as claimed in claims 8 and 9, wherein the administering is carried out intraperitoneally.
- 16) A method of treating Malaria or Surra, said method comprising act of administering 17-allylamino-17-demethoxygeldanamycin (17-AAG) optionally along with at least one pharmaceutically acceptable excipient, to a subject in need thereof.

17) A method of treating Surra, said method comprising act of administering Geldanamycin, optionally along with at least one pharmaceutically acceptable excipient, to a subject in need thereof.

18) The methods as claimed in claims 16 and 17, wherein the administering inhibits Heat Shock Protein 90 (HSP90) of species causing the Malaria and the Surra; and wherein the subject is mammal.

19) The processes as claimed in claims 3, 4, 8 and 9, and the methods as claimed in claims 16 and 17, wherein the excipient is selected from a group comprising gums, granulating agents, binding agents, lubricating agents, disintegrating agents, sweetening agents, coloring agents, flavoring agents, coating agents, plasticizers, preservatives, suspending agents, emulsifying agents, antistatic agents, and spheronization agents or any combination thereof.

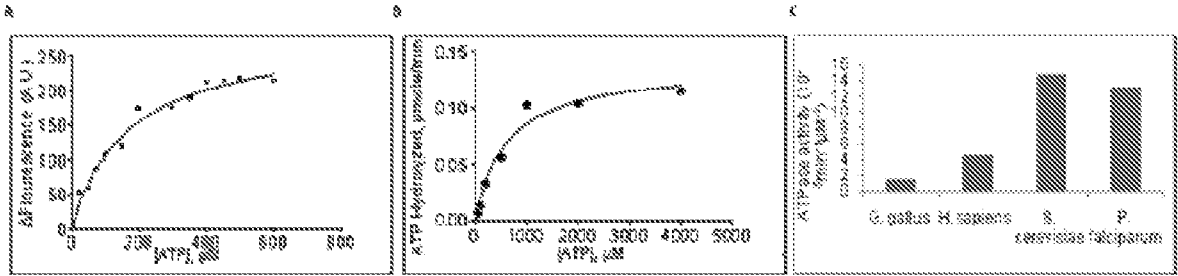


Figure 1

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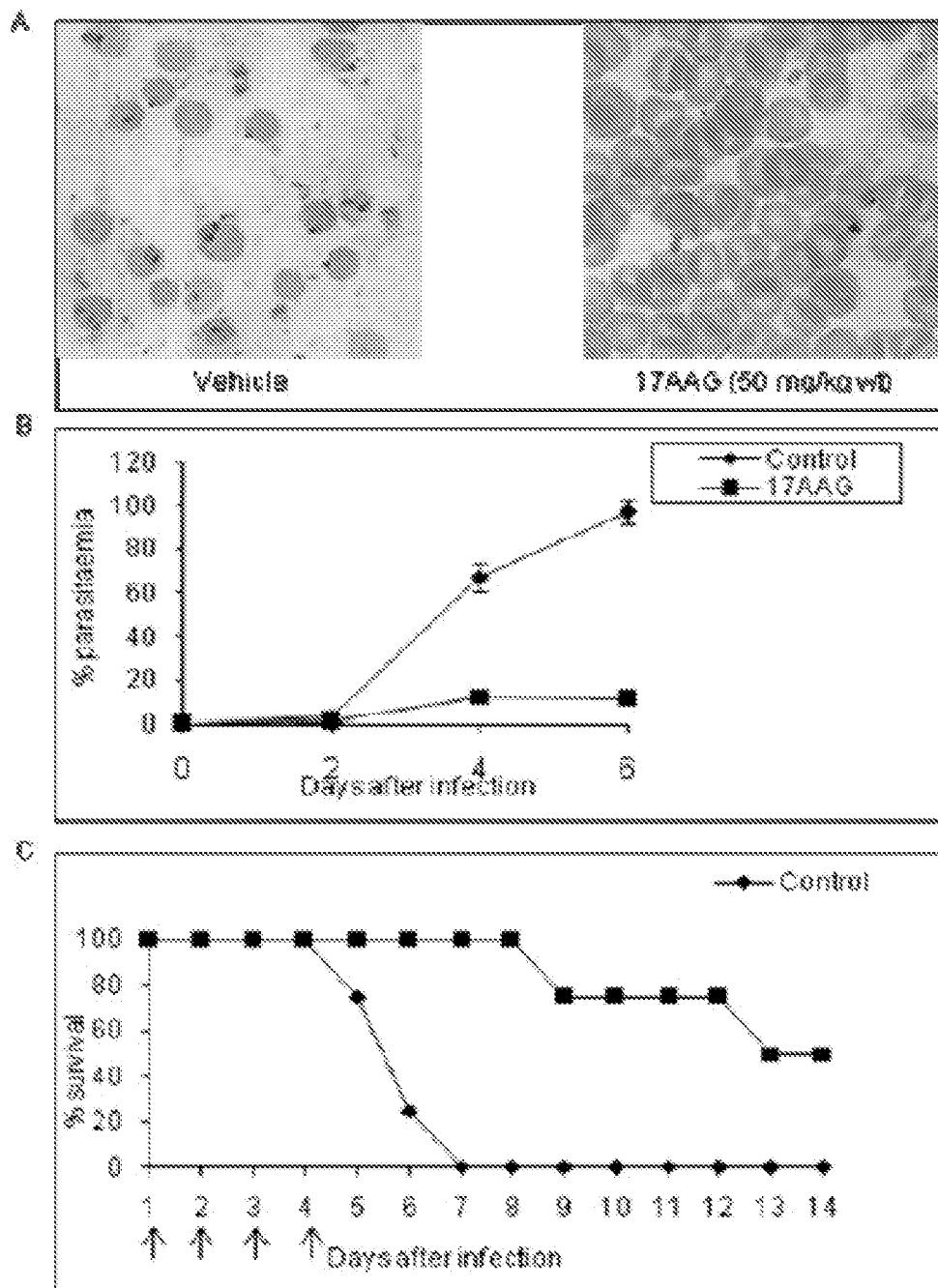


Figure 2

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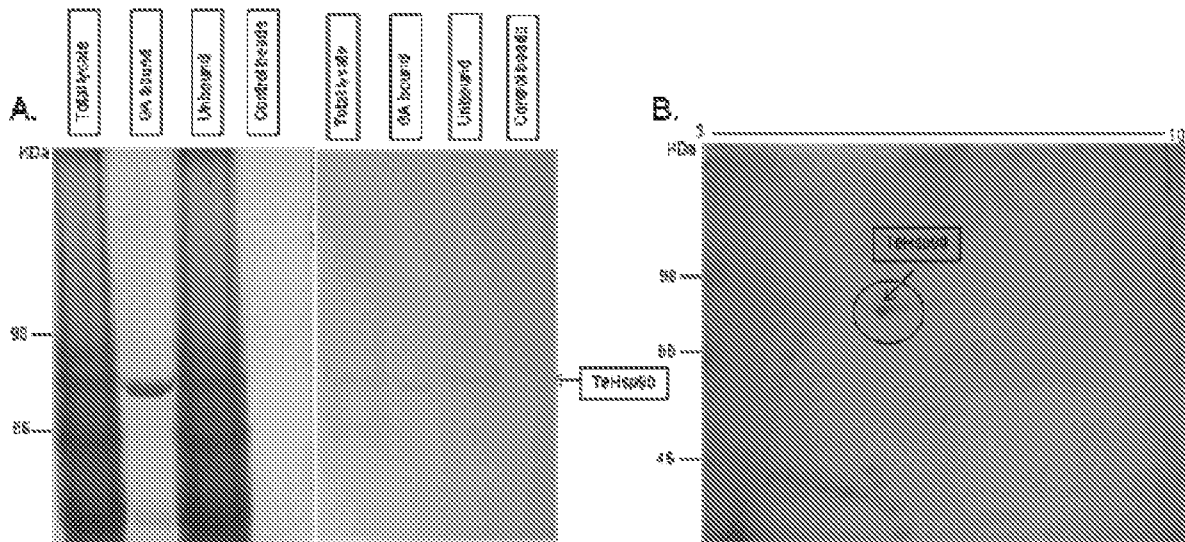


Figure 3

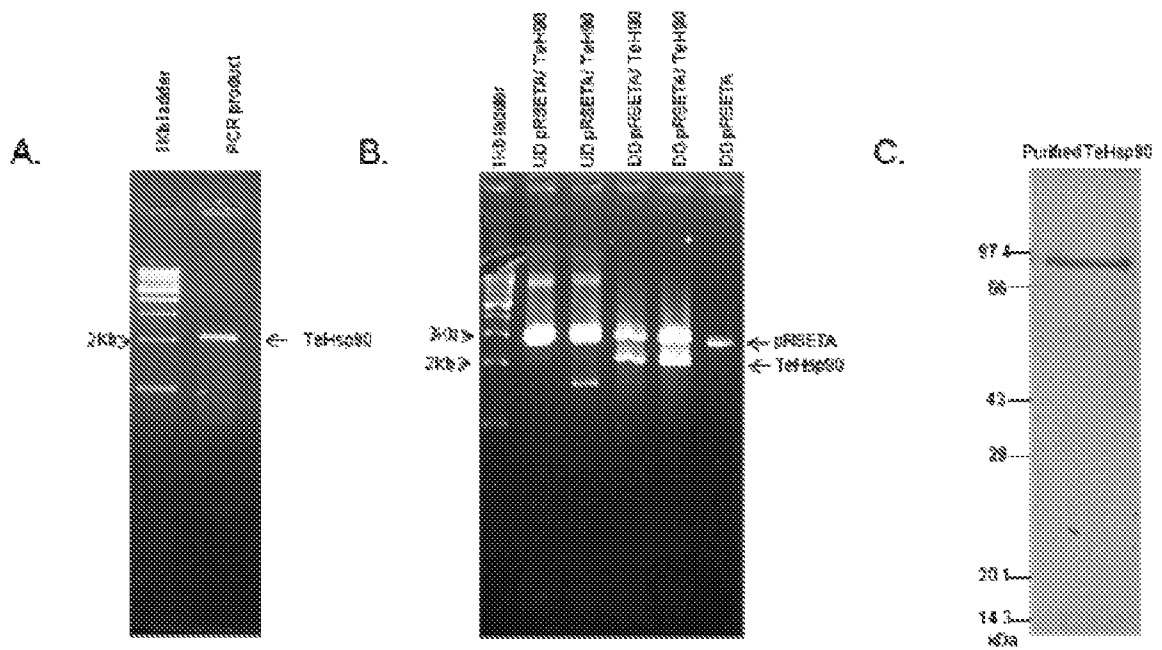


Figure 4

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D.

ATGACGGAGACATTCCGATTCCAGGCTGAAATCAATCAGCTAATGTCCCTTATTATTAATACC  
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 AAAATCCGGTACCAGAGTCTGACGAACCAGTCCGTGCTCGGAGATGAACCCCATTTGCGCAT  
 CCGCTAATCCCCGACCGAGTAAATAAGACACTGACGGTGGAGGACAGTGGCATCGGTATGAC  
 GAAAGCCGACCTGGTGAACAACCTTGGTACGATTGCTCGCTCCGGTACCAAGTCATTATGGA  
 GGCATTGGAGGCGCGGTGGGATATGAGCATGATGGGCGAGTTCGGGTGTCGGATTTTACTCTGC  
 CTACCTTGTGCGCAGACCGCGTAACCGTGTGTGCGAAGAACAACGAGGATGATGCCCTACACGTC  
 GGAATCCTCTGCAGGTGGTACCTTCACTGTAAACCTCCACGCCCGACTGTGACCTGAAGCGCGG  
 TACCGGTATCGTCTTGCAATTGAAGGAAGACCAGCAGGAATACCTGGAGGAGCGCGCGCTGA  
 AGGACCTCATCAAAAAACACTCCGAATTTATCGGCTACGACATCGAGTTAATGGTGGAGAAC  
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 AAGGTGAAGAGCCAAAGGTGGAAGAGGTGAAGGACGGCGATGATGCCGATGCCAAGAAAAA  
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 GCAACAACATCAAGCTATACGTCCGCCCGGTATTTCATCATGGACAACTGTGAGGACCTGTGCC  
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 CACCTCGCTGCTGACATCCGGGTTTACACTTGATGACCCACGGCATAAGCTGACCGTATCCA  
 CCGCATGATCAAAATTGGGTCTCTCACTCGACGATGATGCGGAAGAGGAGGAAGCGCAGGCAC  
 CCGTTGCCGCTGCTGCCGCCAACTCCAGTACTGGTGGTGGGTATGGAGGAAGTGGACTGA

Figure 4

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TeHis90      NTEYFAFQAEINQLNLSLISITFYFWKEIFLRELISHSSDACDEIDYQSLTHQSVLGGSEFN 60
TeHis90      NTEYFAFQAEINQLNLSLISITFYFWKEIFLRELISHSSDACDEIDYQSLTHQSVLGGSEFN 60
*****

TeHis90      LRIIVFIDGQVKKTLFYVDGGIGHTKADLTENLUTTAAGGVTKEFNEALRAGGDHSHIQQFG 120
TeHis90      LRIIVFIDGQVKKTLFYVDGGIGHTKADLTENLUTTAAGGVTKEFNEALRAGGDHSHIQQFG 120
*****

TeHis90      VETFAKTLVADGVTIVKSHKIDAYTHESASGTFVTSTPDCALHGGTKIVLHLEEDQG 180
TeHis90      VETFAKTLVADGVTIVKSHKIDAYTHESASGTFVTSTPDCALHGGTKIVLHLEEDQG 180
*****

TeHis90      EYLEERFLDLIKKHKEFIQYDIELSVLETTKEVTDDEDIEAAKKAEEGEFFVFEVK 240
TeHis90      EYLEERFLDLIKKHKEFIQYDIELSVLETTKEVTDDEDIEAAKKAEEGEFFVFEVK 240
*****

TeHis90      DGGADAPKHTKTKVKEVKEFYVQSHKSLSTEDPEDVKEETASFYELISGGKEQLS 300
TeHis90      DGGADAPKHTKTKVKEVKEFYVQSHKSLSTEDPEDVKEETASFYELISGGKEQLS 300
*****

TeHis90      TENFVDCQLEFFAILFLKRAFTDMEITFEKSHKSHIKYVKKVFTIMCEDLCPEVLS 360
TeHis90      TENFVDCQLEFFAILFLKRAFTDMEITFEKSHKSHIKYVKKVFTIMCEDLCPEVLS 360
*****

TeHis90      FLRGVVDGSDLPLEISPEVILQSHKILKVINGSTVSKALELPERLAEKHEDYERFYEQFN 420
TeHis90      FLRGVVDGSDLPLEISPEVILQSHKILKVINGSTVSKALELPERLAEKHEDYERFYEQFN 420
*****

TeHis90      NFWLGHEDSIENKILKELLAPFESSESGEETITLEDYVTHKEKQKCTTYVTDSEKILE 480
TeHis90      NFWLGHEDSIENKILKELLAPFESSESGEETITLEDYVTHKEKQKCTTYVTDSEKILE 480
*****

TeHis90      TSTFIEQAKPQKHEVLTSDPDEVTNQQVDSFKDCKFACLTKEGVSEETKEENKQEE 540
TeHis90      TSTFIEQAKPQKHEVLTSDPDEVTNQQVDSFKDCKFACLTKEGVSEETKEENKQEE 540
*****

TeHis90      KEASTERLCKAKHEVLQKVFYVVDPLATSPCILVTNEFPQKSHHSHIKRQALSDSS 600
TeHis90      KEASTERLCKAKHEVLQKVFYVVDPLATSPCILVTNEFPQKSHHSHIKRQALSDSS 600
*****

TeHis90      EKATKSHKTHKINTIRAYVELKAPVLAENEDKALDILFLDTSLITSGFTLDGFTA 660
TeHis90      EKATKSHKTHKINTIRAYVELKAPVLAENEDKALDILFLDTSLITSGFTLDGFTA 660
*****

TeHis90      YARRINSHIKLGLSLDDQAKEXAQAFVAAAANSSTGASCHREVD 700
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*****

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Figure 4

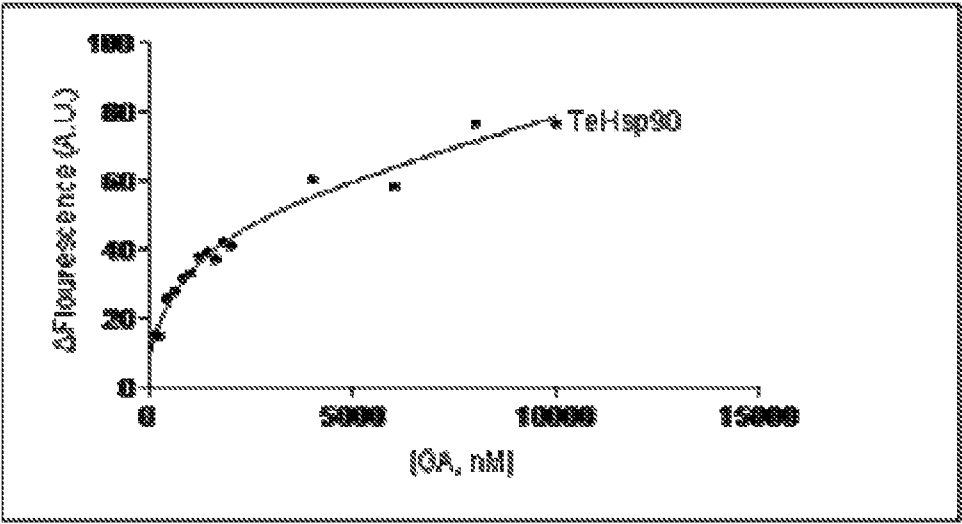


Figure 5

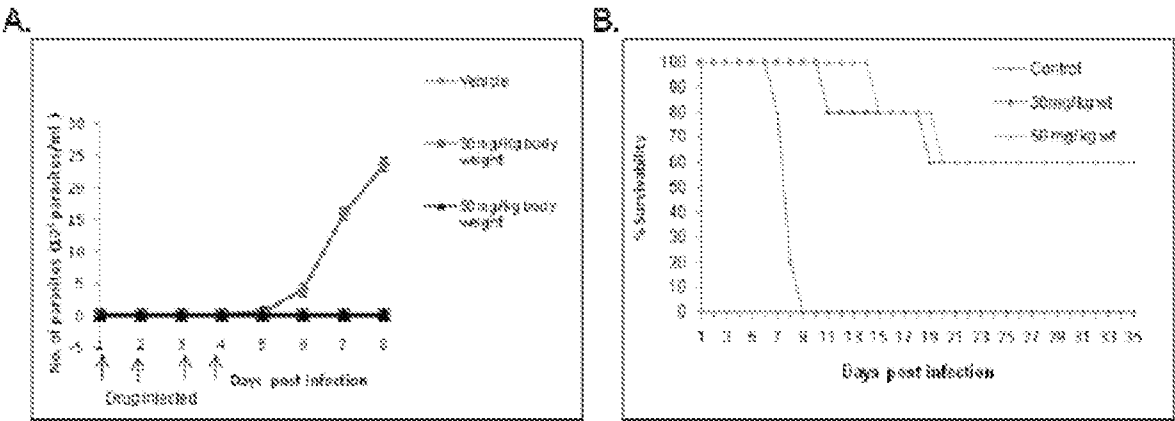


Figure 6