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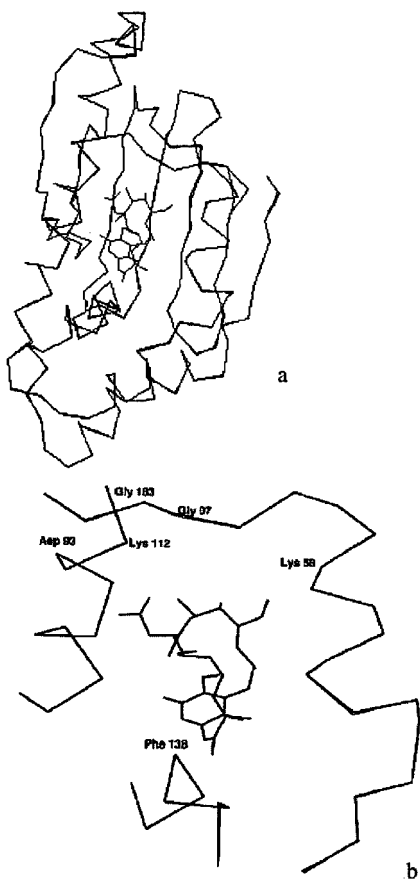
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(54) Title: A NOVEL ASSAY TO SCREEN FOR ANTI-MALARIALS

(57) Abstract: Malarial parasite *Plasmodium falciparum* is responsible for the most severe form of malaria in humans, causing about 2 million deaths every year. Lack of effective vaccines and emergence of drug-resistant strains necessitate the need of novel drug targets to treat the disease. The present invention describes a novel assay method of identifying candidate compounds as anti-malarials based on the property of binding to plasmodial parasite 90 kDa heat shock protein.



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**“A NOVEL ASSAY TO SCREEN FOR ANTI-MALARIALS”**

This invention relates to a novel assay to screen for anti-malarial drugs using plasmodial heat shock protein 90 as target.

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**Prior Art:**

Hsp90 is an abundant protein in eukaryotes comprising of 1-2% of total cellular proteins. It is indispensable for viability of yeast (1). In addition, Hsp90 is also shown  
10 to be essential for growth and development in fruit flies (2) and plants (3). Hsp90 in concert with Hsp70 and other co-chaperones, aids in the folding of certain subset of newly synthesized proteins and helps them to attain their mature, functional conformation. It associates with a variety of substrates in the eukaryotic cytosol like transcription factors (steroid receptors, aryl hydrocarbon receptor, heat shock factor,  
15 mutant p53, etc.), kinases (pp60<sup>v-src</sup>, Raf-1, eIF-2 $\alpha$  kinase, etc.), cytoskeletal proteins (actin and tubulin), telomerase, and proteosomes, thus participating in several important cellular functions like regulation of gene expression, signal transduction, cell proliferation and protein degradation (4, 5).

20 This multi-functional protein has also been shown to take part in protein targeting by associating with different co-chaperones. For instance, it utilizes the co-chaperone p50<sup>cdc37</sup> to transport kinases to plasma membrane and it uses FKBP<sub>s</sub> and p23 to translocate steroid receptors from cytosol to nucleus.

25 Hsp90, in combination with Hsp70 and Hop (Hsp70-Hsp90 organizing protein) forms multi-chaperone machinery in the eukaryotic cytosol (6). This pre-formed complex forms a part of “foldosome”, which helps in the folding and assembly of client proteins. During stress, it prevents misfolding and non-specific aggregation of proteins. It is also involved in the folding of tumor suppressor protein, p53.

30

Hsp90 is found in elevated levels in several tumor cell lines. In tumor cells, Hsp90 stabilizes oncogenic protein kinases, mutated p53, etc. and thus takes part in tumor

cell proliferation (7). Inhibiting Hsp90 function in tumor cells would therefore cause reversion to normal phenotype.

5      Ansamycin antibiotics, Geldanamycin (GA) and Herbimycin A (HA) were isolated from the fungus, *Streptomyces hygroscopicus*. The ansa ring of GA and HA resembles the adenine base of ATP and benzoquinone moiety is analogous to the ribose and phosphate group of ATP. Hence they compete with ATP in binding to ATP-binding domain of Hsp90 (8). GA is known to interfere with Hsp90 function by associating with ATP-binding pocket. When cell extracts were passed through GA-immobilized  
10     column, Hsp90 was the only protein from cell lysates that specifically bound to the column (9,10).

During its asexual life cycle in human erythrocytes, the malarial parasite progresses through three growth phases (11). The early form following invasion, called the ring  
15     stage, is the phase of establishment in the erythrocyte. Trophozoite stage is the metabolically most active, biosynthetic phase while the schizont stage represents the phase of nuclear division before release of merozoites from the erythrocyte. Heat shock proteins of the class Hsp60, Hsp70 and Hsp90 are known to be expressed by the parasite during the intra-erythrocytic stages in the vertebrate host (12,13,14).  
20     While these heat shock proteins share significant homologies with their mammalian counterparts, there is very limited information available about their functional roles in parasite development.

Hsp90 of *Plasmodium falciparum* (PfHsp90) is encoded by a single copy gene located  
25     in chromosome 7. The gene has a single intron of 800 bp and the encoded protein product has 745 amino acids with a molecular weight of 86kDa (15). PfHsp90 sequence is highly conserved across various species. It shows 59% identity and 69% similarity to mammalian Hsp90. Very limited information is available regarding the functional role of PfHsp90 (16,17).

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**Objective of Invention :-**

While it was well known that plasmodial Heat shock proteins of the class Hsp60, Hsp70 and Hsp90 are known to be expressed by the parasite during the intra-erythrocytic stages in the vertebrate host and that these heat shock proteins share significant homologies with their mammalian counterparts, there is very limited information available about their functional roles in parasite development. The objective of the invention was to determine whether these heat shock proteins are functionally important in the growth of parasites *in vivo* and *in vitro* using HSP90 as a model and develop suitable screening assays that will be useful to test chemical library of compounds and select novel anti-malarial agents.

**Summary of Invention :-**

The sequence alignment of *Plasmodium falciparum* HSP90 with human HSP90 ( $\alpha$  and  $\beta$ ) and its homologue Grp94 show that N-terminal domain of PfHsp90 shares 69% identity with human Hsp90. Sequence comparison with human Hsp90 reveals that this domain has an ATP/Geldanamycin binding site. The GXXGXXG motif in this domain essential for ATP-binding is present in PfHsp90 also. The residues, which make contact with GA in mammalian Hsp90, are also conserved in PfHsp90. Both Geldanamycin and Herbimycin A kill the malarial parasite at the stage of early trophozoite development thereby showing that compounds binding to the geldanamycin binding site can be potential anti-malarial agents. It has been shown in *in vitro* assays that geldanamycin binds to *Plasmodium falciparum* HSP90 and such binding can be quantified using suitable immunochemical or radiochemical or non-radioactive assays. It has therefore been possible to describe in the assays wherein any suitably immobilized compound can be tested for the potential to bind to Plasmodium HSP90; the specific anti-malarial effect of a compound that binds to Plasmodium HSP90 can then be tested using known methods. Such assays can be further developed into high throughput assays using the currently known technologies enabling screening of compound libraries as exemplified by the BIAcore assay.

**DETAILED DESCRIPTION OF THE INVENTION:**

A prototype assay is described below:

- 5 1.0: Covalent immobilization of the test compound on suitable matrices such as Sepharose or BIAcore **CM5** sensor chips by known methods.
- 2.0:** Preparation of Saponin-freed Plasmodial trophozoite lysate. *P. falciparum* infected erythrocytes in the trophozoite stage were treated with a final concentration
- 10 of **0.075%**saponin in PBS (10mM sodium phosphate (pH 7.4), **137mM** NaCl, **2.7mM** KCl.) and centrifuged at **4,500** rpm for **4** min. Parasites are in the pellet fraction and are then washed twice in PBS and lysed in TNESV buffer (**50 mM** Tris-HCl, (pH **7.5**), **1%NP-40**, **2 mM** EDTA, **100 mM** NaCl, **1 mM** orthovanadate).
- 15 3.0: Reacting the trophozoite lysate with the covalently immobilized test compound.
- 4.0:** Detection of the compound bound *Plasmodium falciparum* 90 kDa heat shock protein. This can be performed either by western blotting using polyclonal antibodies to PfHsp90 or by use of radiolabeled trophozoite protein extracts and subsequent
- 20 detection by phosphorimager analysis as described for analysis of GA binding in examples **3.1** to **3.4**. All compounds showing more than **10%** of total binding to PfHsp90 were used for further screening.

Known Covalent Immobilization methods are:

- 25 1. Photochemical coupling.
- 2.** Covalent immobilization of biomolecules using beads coated with carboxyl groups (e.g., LiquiChip Carboxy Beads, Qiagen) using carbodiimide chemistry. In this process, beads are washed and incubated with an activator (typically EDC/NHS), washed again, the capture molecule is added and incubated, and the beads washed
- 30 again.
- 3.** Using immobilized PCR primers
- 4.** Spatially controlled covalent immobilization of Biomolecules on silicon surfaces.

In addition, a non-radioactive assay for screening test compound and related compounds using SPR analysis with a BIAcore 2000<sup>TM</sup> (Amersham Biosciences) biosensor system may also be used based on technical details provided for GA binding in example 3.4. Briefly, test compound is covalently immobilized on the

5 research **CM5** sensor chips at a concentration of 20 **mM** in 8% **DMSO** using the amine coupling kit (**1-ethyl-3-(dimethylaminopropyl)** carbodiimide), (N-hydroxysuccinimide) provided by the manufacturer. For compounds of unknown structure, derivatization is done with biotin using photobiotin acetate (22) followed by BIAcore analysis using the BIAcore SA chip that carries a streptavidin surface.

10 All measurements are carried out with TNESV buffer (50 mM Tris-HCl, (pH 7.5), 1% NP-40, 2 mM EDTA, 100 mM NaCl, 1 mM orthovanadate). The surface regenerated by a 50 s pulse of 0.5% **SDS** flowing at **10  $\mu$ L/min**. For binding analysis, saponin released trophozoites of *P. falciparum* are lysed in an equal volume of TNESV buffer and the lysate is clarified by centrifugation at 20,000 g for 20 min.

15 Binding is evaluated by passing the parasite lysate at a flow rate of 1  $\mu$ L/min in TNESV buffer and measuring the change in refractive index as was done for GA binding in example 3.4.

Compounds thus selected by any of these assays or a combination thereof can then be

20 further tested using known assays described in examples 2.1 and 2.2 and the novel flow cytometry assay as described in example 3.5 to select for the desired anti-malarial effect.

### **Examples:**

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#### **Example 1.0 : Geldanamycin binding site in Pf HSP90**

Sequence alignment of Human Hsp90 ( $\alpha$  and  $\beta$ ) and its homolog **Grp94** with PfHsp90 indicates that several domains are evolutionarily conserved suggesting functional

30 similarity among Hsp90 proteins of different organisms. PfHsp90 possesses a charged acidic domain in the central region, which is absent in human Hsp90. N-terminal domain of PfHsp90 shares 69% identity with human Hsp90. Sequence comparison with human Hsp90 reveals that this domain has an ATP/Geldanamycin

binding site. The GXXGXXG motif in this domain essential for ATP-binding is present in PfHsp90 also. The residues, which make contact with GA in mammalian Hsp90, are also conserved in PfHsp90.

5 **Crystal** structure of N-terminal Hsp90 - GA complex has already been solved (PDB ID: 1YET) (18). High degree of similarity between the ATP-binding domains of mammalian Hsp90 and PfHsp90 enabled us to model the ATP-binding domain of PfHsp90 using mammalian Hsp90 as a template. N-terminal sequence of PfHsp90 (1-177 amino acids) was submitted to SWISS-MODEL program to obtain the 3-D  
10 structure using the crystal structure of N-terminal Human Hsp90 (PDB code: 1YET) as a template. This model was superimposed over the structure of human Hsp90-GA complex using STAMP (Structure Alignment of Multiple Protein) program as shown in Fig. 1a of the accompanying drawings. PfHsp90 is shown in blue line while yellow line denotes mammalian Hsp90. GA is represented in yellow colour in the centre of  
15 the structure.

Fig. 1b shows a magnified view of the GA-binding region and the contact making residues, namely Lys 58, Asp 93, Gly 97, Lys 112, Phe 138 and Gly 183 were indicated. Sequence and structure comparison suggests that GA could possibly  
20 interact with PfHsp90 also.

## **Example 2.0 : Effect of ansamycin antibiotics on Plasmodial parasite.**

### **2.1 : Growth inhibitory effect of GA:**

25

In order to check if GA could inhibit *Plasmodium falciparum* growth *in vitro*, we prepared highly synchronous ring-infected parasites using 5% sorbitol. These ring-staged parasites were suspended in RPMI to make 5% hematocrit. 200µl cell suspension was treated with DMSO or with GA in DMSO at various concentrations  
30 (0.5, 1, 5 and 10 µM) for 24h. Smears were taken at the end of treatment, Giemsa-stained and viewed under microscope. While the DMSO treated rings progressed to trophozoite stage at the end of 24 hrs, GA-treated parasites persisted in the ring stage even after 24 hrs. The degree of inhibition in stage progression increased with



increasing concentration of GA. Maximal growth inhibition was found at 5  $\mu\text{M}$  concentration. The same experiment was performed with early-trophozoites and schizonts also. When GA was added in the trophozoite stage, 100% growth inhibition was observed at a GA concentration of 10  $\mu\text{M}$ . This correlates with elevated expression of PfHsp90 in the trophozoite stage compared to ring stage. When schizont-staged parasites were treated with GA, it did not affect the release of merozoites and their subsequent reinvasion of erythrocytes.

## **2.2 : Anti-malarial effect of GA and HA**

10

To show the effects of GA on overall parasitemia, ring-infected erythrocytes were either treated with DMSO or with 5  $\mu\text{M}$  GA for 48hrs (culture was replenished with medium and GA every 24hrs) and parasitemia was calculated by giemsa staining. While the mock-treated parasites multiplied at the end of 48 hrs, parasitemia was reduced to 50 percent in GA treated cultures by 48 hrs. When cultures were similarly treated with various concentrations of HA (0.5, 1, 5 and 10  $\mu\text{M}$ ), maximal reduction in parasitemia was observed at 10 $\mu\text{M}$  concentration.

Examples 2.1 and 2.2 clearly demonstrate that ansamycin antibiotics affect overall parasitemia at the stage of the formation of trophozoites.

## **Example 3.0 : In vitro Assay to test the effect of geldanamycin for binding to PfHsp90 and parasite growth inhibition**

### **3.1. :Solid-phase GA binding assay:**

Geldanamycin was immobilized on sepharose beads (9). GA was first modified by adding 1,6- hexanediamine as a linker for coupling to the activated ester beads. 1,6-hexanediamine was added to GA (10 mM in  $\text{CHCl}_3$ ) to yield 10-fold molar excess and allowed to react at room temperature for 2 h in the dark to yield 17-hexamethylenediamine-17-demethoxy-geldanamycin. The reaction solution was extracted with water to remove the unreacted 1,6- hexanediamine. The products were then dried under nitrogen, redissolved in DMSO, and reacted with NHS-activated

Sepharose 4 fast flow beads (Amersham Biosciences) for 2 h in the dark at room temperature. The resulting beads were washed twice in ice-cold TNESV buffer (50 mM Tris-HCl, (pH 7.5), 1% NP-40, 2 mM EDTA, 100 mM NaCl, 1 mM orthovanadate) and rocked overnight at 4°C. GA beads were then blocked with 1% BSA for 1 h. For control beads, the resin was treated in the same way, but without adding 17-hexamethylenediamine-17-demethoxy-geldanamycin.

### **3.2 :Immunoblot analysis of PfHSP90 and GA binding:**

10 *P. fakiarum* cultures were treated with saponin at a concentration of 0.075% to release the trophozoites. Saponin released trophozoites of *P. fakiarum* were lysed in TNESV buffer (50 mM Tris-HCl, pH 7.5), 1% NP-40, 2 mM EDTA, 100 mM NaCl, 1 mM orthovanadate) containing protease inhibitors. Clarified lysate was incubated with either with GA coupled beads or control beads and incubated overnight at 4 °C.

15 Beads were washed four times with TNESV buffer, solubilized in Laemmli buffer and analyzed on SDS-PAGE (7.5% resolving gel with 3% stacker gel) (19). Proteins were transferred to nitrocellulose membrane and the membrane was blocked in 1% milk powder in TBST (20 mM Tris, 136 mM NaCl, 0.05% Tween-20, pH 7.2) for 1h. The membrane was then washed thrice with TBST for 10 min each. The membrane was

20 then probed with rabbit polyclonal antibody to PfHsp90. After 3 washes with TBST for 10 min each, the membrane was probed with anti-rabbit IgG conjugated to ALP (alkaline phosphatase). The membrane was developed using BCIP (5-bromo, 3-chloro, indolyl phosphate)/NBT (nitroblue tetrazolium) as substrate. Under these conditions, we could clearly see binding of PfHsp90 to the GA-coupled beads.

25 Control beads did not show any binding to PfHsp90.

### **3.3 :Analysis of PfHSP90 and GA binding to the protein**

*P. fakiarum* infected erythrocytes in the trophozoite stage were labeled with [<sup>35</sup>S]-cys and [<sup>35</sup>S]-met. Following lysis with 0.075% saponin, parasites were lysed in TNESV buffer containing protease inhibitors. Lysate was incubated with either with GA coupled beads or control beads and tumbled overnight at 4 °C. Beads were washed four times with TNESV buffer and solubilized in 2D lysis buffer. The sample

in 2D lysis buffer was loaded onto 7 cm x 1.5 mm **IEF** tube gels pre-focused at 250 V for 30 mins. Following this, the tube gels were electrophoresed at 500 V for 4 hours. After the run was over, the tube gels were incubated in 1 ml of equilibration buffer (125 mM Tris (pH 6.8), 4.9 mM DTT, 10% glycerol, and 2% SDS, pH 6.8) for 9  
5 minutes. The tube gels were laid horizontally on top of 7.5% SDS-polyacrylamide gels and sealed with 1% agarose in SDS-PAGE **running** buffer (50 mM Tris, 380 mM glycine, and 0.1% SDS). SDS-PAGE was carried out at 110 V for one hour and 10  
10 minutes. The spots were visualized using fluorography (20). Earlier work from the lab has unequivocally established the position of PfHsp90 (pI = 4.94, M<sub>w</sub> = 86 kDa) on  
2D-PAGE (21). Based on this, the protein bound to GA coupled beads was identified as PfHsp90. Control beads did not show any binding to PfHsp90.

#### **3.4 : Analysis of PfHSP90 and GA binding by Surface Plasmon Resonance Spectroscopy:**

15

To test the feasibility of a non-radioactive assay for screening GA and related compounds, we used SPR analysis with a BIAcore 2000™ (Amersham Biosciences) biosensor system. Geldanamycin was covalently immobilized on the research CM5 sensor chips at a concentration of 20 mM in 8% DMSO using the amine coupling kit  
20 (1-ethyl-3-(dimethylaminopropyl) carbodiimide, N-hydroxysuccinimide) provided by the manufacturer. Nearly 700 resonance units (RU) of GA were immobilized under these conditions. The unreacted moieties on the surface were blocked with 1M ethanolamine. All measurements were carried out with **TNESV** buffer. The surface was regenerated by a 50-s pulse of 0.5% SDS flowing at 10 μL/min.

25

For binding analysis, saponin released trophozoites of *P. falciparum* were lysed in an equal volume of **TNESV** buffer and the lysate was clarified by centrifugation at 20,000 g for 20 min. Binding was evaluated by passing the parasite lysate at a flow rate of 1 μL/min in **TNESV** buffer and measuring the change in refractive index **as**  
30 response units. Upon passing the parasite lysate over the GA coupled chip, significant binding (response of 900 RU) was observed. In order to make sure that the binding was due to PfHsp90, parasite lysate was mixed with antiserum to PfHsp90 and then passed over the GA coupled chip. In this instance, binding was completely abolished,

indicating that PfHsp90 from the parasite lysate was specifically binding to GA coupled in the chip.

### **3.5 : Analysis of the inhibition of *P. falciparum* growth by GA using flow**

#### **5 cytometry**

In order to confirm *P. falciparum* inhibition by GA and related compounds, we developed an assay involving flow cytometry. Briefly, parasites were purified on 75% percoll thrice in order to obtain a pure parasite population free of uninfected RBCs.

10 30  $\mu$ L of such a suspension was added to 600  $\mu$ L of the staining solution (6 mg/L of acridine orange in 10mM Tricine and 120mM  $\text{NaH}_2\text{PO}_4$  at pH 9). The suspension of stained cells was injected into a flow cytometer (BD (Becton Dickinson) FACScan) equipped with an argon ion laser set at 488 nm at a power output of 15 mW. Green fluorescence (GF) was detected at 560 nm. Side scatter (SSC) was detected

15 simultaneously with GF. At most, 10,000 cells were assessed and plotted in a two-dimensional scattergram of SSC (linear scale) against GF (logarithmic scale) in less than 60 seconds. The parasite area was defined in a two dimensional scattergram of SSC vs. GF. The **ring** and trophozoite forms were clearly defined by analysis of scattergrams for parasites at various times after synchronization. The validity of the

20 assay was confirmed by treating highly synchronous **ring** stage parasites with 10  $\mu$ M GA for 24 h. DMSO treated parasites were used as control. GA treated parasites displayed a scattergram corresponding to **rings** while the scattergram of the DMSO treated control corresponded to that of trophozoites. The data clearly demonstrates that GA and related compounds can be effective as anti-malarials.

25

Based on the assays described in example 3 of this specification, it is feasible for one skilled in the **art** to develop similar assays and to test for the binding of test compounds to PfHsp90 (using solid phase coupling to sepharose beads and BIACORE chips) **as** well as for inhibition of the growth of *P. falciparum* (using the flow

30 cytometry assay).

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20 **600.**

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**Claims :**

1. A novel assay to screen for anti-malarial drugs by testing for the binding of the test compound with plasmodium 90kDa heat shock protein which comprises:
  - 5 a) Covalent immobilization of the test compound on suitable matrices such as Sepharose or BIAcore CM5 sensor chips by known methods;
  - b) Preparation of saponin-freed Plasmodial trophozoite lysate by known methods;
  - 10 c) Reacting the trophozoite lysate with the covalently immobilized test compound, and
  - d) Detection of the compound bound Plasmodial 90kDa heat shock protein by known methods.
  
2. A novel assay as claimed in claim 1, wherein the compound bound Plasmodial  
15 90 kDa heat shock protein is detected by known immunochemical methods.
  
3. A novel assay as claimed in claim 1, wherein the compound bound Plasmodial  
20 90 kDa heat shock protein is detected by known radiometric methods such as 2D gel electrophoresis and fluorography.
  
4. A novel assay as claimed in claim 1, wherein solid phase matrix such as BIAcore 2000<sup>TM</sup> is used to perform the assay.
  
5. A novel assay as claimed in claim 4, wherein the assay performed on solid  
25 matrix such as BIAcore 2000<sup>TM</sup> comprises of the following steps:
  - a) Compounds that can be derivatized with amine groups are covalently immobilized on the research CM5 sensor chips at a concentration of 20 mM in 8% DMSO using the amine coupling kit (1-ethyl-3- (dimethylaminopropyl) carbodiimide, (N-hydroxysuccinimide);
  - 30 b) The unreacted moieties on the surface being blocked with 1M ethanolamine;
  - c) The surface was regenerated by a 50 s pulse of 0.5% SDS flowing at 10µL/min;



- d) Saponin released trophozoites of *P. falciparum* being lysed in an equal volume of TNESV buffer for binding analysis;
- e) The lysate being clarified by centrifugation at 20,000 g for 20 min. and
- f) Binding is evaluated by passing the parasite lysate at a flow rate of 1 $\mu$ L/min in
- 5 TNESV buffer and measuring the change in refractive index as response units.
6. A novel assay as claimed in any one of the preceding claims, wherein for compounds of unknown structure, derivatization is done with biotin using photobiotin acetate followed by BIAcore analysis using the BIAcore SA chip that carries a
- 10 streptavidin surface.

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Fig. 1a

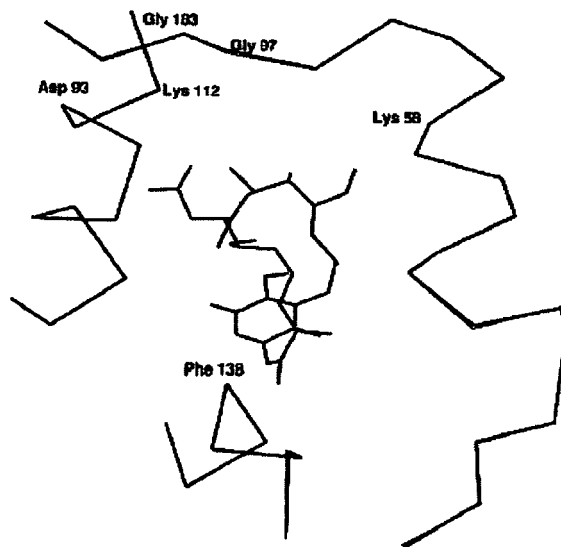


Fig. 1b

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/IN 03/00374-0

## CLASSIFICATION OF SUBJECT MATTER

PC<sup>7</sup>: G01N 31/00, G01N 33/00 // C07K 14/445

According to International Patent Classification (IPC) or to both national classification and IPC

## 3. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

PC<sup>7</sup>: G01N, C07K

Minimum documentation searched (classification system followed by classification symbols) to the extent that such documents are included in the

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MPI, EPODOC, CAPLUS, MedLine

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Grenert J.P., et al. "The Amino-terminal Domain of Heat Shock Protein 90 (hsp90) That Binds Geldanamycin Is an ATP/ADP Switch Domain That Regulates hsp90 Conformation". The Journal of Biological Chemistry, 1997, Vol. 272, No. 38, pp. 23843-23850 <i>the whole document.</i>	1-6
A	Gowrishankar Banumathy, et al. "Heat Shock Protein 90 Function Is Essential for Plasmodium falciparum Growth in Human Erythrocytes". The Journal of Biological Chemistry, 2003, Vol. 278, No. 20, pp. 18336-18345 <i>the whole document.</i>	1-6
A	Rajinder Kumar, et al. "The heat shock protein 90 of Plasmodium falciparum and antimalarial activity of its inhibitor, geldanamycin". Malaria Journal, 15. 09. 2003, 2:30, pp. 1 November 2004 (01.11.04) <i>the whole document.</i>	1-6

 Further documents are listed in the continuation of Box C. See patent family annex.

\* Social categories of cited documents:

„A“ document defining the general state of the art which is not considered to be of particular relevance

„E“ earlier application or patent but published on or after the international filing date

„L“ document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

„O“ document referring to an oral disclosure, use, exhibition or other means

„P“ document published prior to the international filing date but later than the priority date claimed

„T“ later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

„X“ document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

„Y“ document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

„&amp;“ document member of the same patent family

Date of the actual completion of the international search

18 February 2004 (18.02.2004)

Date of mailing of the international search report

10 March 2004 (10.03.2004)

Name and mailing address of the ISA/AT

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**INTERNATIONAL SEARCH REPORT**

International application No.

**PCT/IN 03100374-0**

(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with date where appropriate, of the document passages	Relevant to claim No.
P,A	NO 03/050295 A2 (Conforma Therapeutics Corporation) 19 June 2003 (19.06.03) <i>the whole document.</i> -----	1-6

## Supplemental sheet

2) In claims 1, 4, 5 and 6 trademarks and/or brand names are used. Such trademarks and/or brand names are not allowable in claims, and should be replaced by accurate chemical and/or technical terms.

## Examples:

- ) Claim 1: Sepharose; BIAcore CM5
- ) Claim 4: BIAcore2000
- ) Claim 5: BIAcore2000; CM5 sensor chips; TNEVS buffer
- ) Claim 6: BIAcore analysis; BIAcore SA chip

1) The following broad and unclear expressions in the claims should be replaced/defined by accurate technical/chemical terms:

- ) Claim 1: "suitable matrices"
- ) Claim 5: "solid matrix"
- ) Claim 5: "amine coupling kit"
- ) Claim 5: "TNEVS buffer"

3) Expressions in parentheses should be removed from the claims.

# INTERNATIONAL SEARCH REPORT

## Information on patent family members

International application No.

PCT/IN 03/00374-0

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
A		none	