Hsp90 Inhibitors in Parasitic Nematodes: Prospects and Challenges

Running title: Hsp90 in parasitic nematodes

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Abstract

Hsp90 inhibitors are well characterized in relation to their effects in a variety of tumors, with several inhibitors in various phases of clinical development. In recent years, the same inhibitor classes have been tested for efficacy in other systems, such as Alzheimer's disease and a variety of infectious disease models, including fungal and parasitic targets. In this article we discuss the repurposing of Hsp90 inhibitors for parasitic disease with a focus on parasitic nematode infections. We summarize the data that indicates that Hsp90 is functionally diverse in different nematode species and we discuss the challenges and prospects for developing these inhibitors as next generation chemotherapeutic tools.
Key words: parasitic nematode; *Brugia pahangi*; *Caenorhabditis elegans*; drug resistance; Hsp90 inhibitors.

Why target Hsp90?

All cells express a suite of proteins known as heat shock proteins (Hsps), which act as molecular chaperones, participating in the folding/refolding of newly synthesized or denatured proteins, as well as facilitating survival following heat shock. Of the heat shock protein family, Hsp90 is the best characterized because of its diverse functions in the biology of the cell (reviewed in [1]). Proteins that require Hsp90 function for their activity are known as 'client' proteins and the Picard laboratory curates an ever-increasing database of these proteins (see http://www.picard.ch/downloads/Hsp90interactors [2]. Amongst these are a sub-set of proteins with essential roles, including many involved in signal transduction pathways. The realization that many Hsp90 client proteins were over-expressed in tumor cells led to an explosion of activity in cancer biology, with many laboratories seeking to develop new therapeutics based on the inhibition of Hsp90 [3]. Hsp90 is an attractive target in tumor cells because inhibiting the function of this one protein affects multiple client proteins and, consequently, the networks in which they interact.

Most inhibitors described to date bind in the nucleotide pocket at the N-terminus of Hsp90 i.e. they block the ATPase cycle, resulting in the destabilization of the client cargo. Inhibitors, such as geldanamycin (GA) and radicicol (RAD) are natural products, as is the C-terminal inhibitor novobiocin. These have provided the templates for a wide variety of synthetic scaffolds [4]. There is vast literature on Hsp90 in malignant cells describing *in vitro* and *in vivo* models and clinical efficacy of inhibitors and for further information the reader is referred to recent reviews (see for example, [5, 6]). As well as their efficacy in tumor cells, Hsp90 inhibitors have been explored as therapeutic tools in other human diseases, particularly neurodegenerative conditions such as Alzheimer's disease [7] and in a variety of infectious diseases.

Hsp90 is an essential protein in all eukaryotes, and the relative effect of inhibitors on tumor cells versus non-transformed cells has presented something of a conundrum: in many cell types, both transformed and non-transformed, Hsp90 represents in the order of 1-2% of total cytoplasmic protein, so why do tumor cells show a greater susceptibility to Hsp90 inhibitors than do normal cells? The first clues as to the differential effect of Hsp90 inhibitors on malignant versus non-malignant cells came from the studies of Kamal et al [8], who used a competitive binding assay to demonstrate that tumor cell Hsp90

had a greater affinity for GA than did Hsp90 from normal cells. That study revealed that most of the Hsp90 in tumor cells was present in complexes containing co-chaperones, in contrast to that from normal cells, where most Hsp90 was uncomplexed. By *in vitro* reconstitution it was demonstrated that complexed Hsp90 had an increased binding affinity for inhibitor compared to the latent form, results that were recapitulated *in vivo* by comparison of malignant versus normal tissues. Subsequent studies confirmed that in tumor cells Hsp90 exists in multi-chaperone complexes with a high affinity for certain inhibitors. For example, proteomic analysis of pull-downs using the purine scaffold compound PU-H71 to isolate Hsp90 complexes produced a snapshot of the global Hsp90 interactome in malignant cells. That study revealed the presence of numerous co-chaperones and oncoproteins complexed to PU-H71-bound Hsp90, in contrast to that precipitated using an anti-Hsp90 antibody [9]. Such studies help explain the increased affinity of inhibitors for Hsp90 complexes in tumor cells, as well as exemplifying the multiple signaling pathways in which Hsp90 interacts. The in-depth understanding of this phenomenon in cancer cell biology has stimulated interest in other fields: could other organisms express high-affinity Hsp90 complexes that are more susceptible to inhibitors? A better understanding of this aspect of Hsp90 biology might help determine if it is also a good target in parasitic organisms.

Hsp90 in parasites

As their name implies, Hsps are frequently induced upon exposure to increased temperature, helping to maintain homeostasis within the cell. Exposure to fluctuating temperatures is an important feature of the life cycle of many parasitic organisms that are transmitted from ambient environmental temperatures to the body temperature of a mammalian host. These transitions are often accompanied by a heat shock response, which presumably facilitates survival and infection of the new host [10]. Hsp90 is a highly conserved molecule and has been identified in many parasitic protozoa (single cell organisms) and helminths (multicellular worms). It is probably best characterized in protozoa such as the apicomplexan parasites, *Plasmodium falciparum* and *Toxoplasma gondi* and the trypanosomatids, *Trypanosoma spp.* and *Leishmania spp.* Protozoan Hsp90s have been the subject of several recent reviews (see for example [11]), and are not covered in detail here. In *T. gondii* many of the proteins interacting with Hsp90 have been identified by pull-down or predicted from bioinformatics analyses, while experimental studies have implicated Hsp90 in both replication and cell invasion (reviewed in [12]). In *Leishmania donovani*, Hsp90 has a unique role in differentiation; this parasite is transmitted to

the mammalian host by the bite of a sandfly and exposure of the promastigote (fly stage) to Hsp90 inhibitors, such as GA, triggers differentiation to the amastigote (mammalian stage), implicating Hsp90 in the regulation of the life cycle in *L. donovani* [13]. However, conversion of *Leishmania major* from promastigote to amastigote cannot be induced with GA [14]. Much of the emphasis on Hsp90 in protozoan parasites has focused on its potential as a drug target [15], as there is a pressing need for novel therapeutics to combat drug resistant strains of the malaria parasite, *P. falciparum*, and to target neglected tropical diseases such as human African trypanosomiasis (or sleeping sickness), caused by *Trypanosoma brucei*. Studies in mouse models of malaria infection have shown synergy between existing anti-malarial compounds, such as chloroquine, and Hsp90 inhibitors [16], while recent work has identified structural differences between *P. falciparum* and human Hsp90 that could be exploited for drug design [17]. An *in silico* structure-activity screen identified compounds that bound preferentially to the parasite Hsp90. Using yeast strains transfected with either parasite or human *hsp90*, the differential susceptibility of *P. falciparum* Hsp90 to the 7-azaindole compounds was confirmed. Drug repurposing is a popular concept for tropical diseases and screening of existing libraries of Hsp90 inhibitors identified compounds with preferential effects on *T. brucei* Hsp83 (the *T. brucei* orthologue of *hsp90*) compared to human Hsp90 [18].

In parasitic worms, other than nematodes, Hsp90 has been partially characterized in *Schistosoma* spp. the blood flukes that cause schistosomiasis, another important neglected tropical disease. Hsp90 was first cloned from *S. mansoni* by screening cDNA libraries with antibodies from infected patients, reflecting its relative abundance/antigenicity [19]. Schistosomiasis infects around 200 million individuals globally; there is no vaccine and control is largely based on the use of a single drug, praziquantel. Although praziquantel is a very safe and effective therapeutic, some field isolates of *S. mansoni* show reduced susceptibility, and praziquantel resistance can be selected for in the laboratory (reviewed in [20]), underscoring the desirability of developing alternative therapies. Schistosomes are susceptible to Hsp90 inhibitors and *in vitro* exposure of adult *S. japonicum* to derivatives of GA is lethal [21]. These parasites are transmitted to humans via contact with water contaminated by the fresh water snail intermediate host and thus provide another example of a parasite that transits from ambient to mammalian body temperature during the life cycle. The infection of snails appears to be a temperature-sensitive process and heat shock of otherwise resistant snails confers susceptibility to

infection, a phenomenon that was reversed by exposure of snails to GA [22]. However, most research on Hsp90 in parasitic helminths has focused on nematodes, and the role of Hsp90 in these organisms is discussed below.

Nematode parasites and the need for novel drugs

Nematodes, or roundworms, are a large phylum of organisms that have evolved to inhabit most ecological niches, including humans and animals. Their success is reflected in their ability to adapt to diverse environments and they can be found from the Antarctic to hot springs. In the order of 25,000 nematode species are known, with many more thought to be as yet unidentified, and while the vast majority are free-living, those that have adopted a parasitic life cycle are of particular importance as pathogens of humans and animals. In humans, the so-called soil-transmitted helminths are the most numerous, infecting many hundreds of millions of individuals mostly in the tropical zones of the world [23]. Adult worms live in the gastro-intestinal tract and comprise three major species, *Ascaris lumbricoides* (the large roundworm), *Trichuris trichiura* (the whipworm of the large intestine) and *Ancylostoma duodenale* and *Necator americanus* (the hookworms). These nematodes often occur in combination and are important pathogens in the tropics, particularly in children [24]. There are no vaccines and their control is completely dependent upon the use of drugs such as the benzimidazole compounds and ivermectin. Interestingly, both classes of drug were originally repurposed from veterinary medicine [25]. The filarial worms are an additional group of tropical pathogens that are transmitted to humans by the bite of an insect vector. The most wide spread of these are the lymphatic filarial worms, *Wuchereria bancrofti* and *Brugia malayi* or *B. timori*. These, and the related parasite *Onchocerca volvulus*, are tissue dwelling species that can survive in the human host for many years and provoke a range of pathologies [26, 27]. Control of filarial worms depends largely upon on killing the larval stage (the microfilaria, Mf) in the skin (*O. volvulus*) or blood (lymphatic filariae) using either ivermectin or diethylcarbamazine (DEC) an older compound, the precise mode of action of which is still open to debate [28]. Although both ivermectin, and particularly DEC, can affect the viability of adult worms, they have to be administered over prolonged periods of time to achieve this effect. Thus at present, control relies upon clearance of Mf by the annual administration of DEC or ivermectin, administered together with albendazole, over the long reproductive life span of the adult worm, which can be approximately 8 years for lymphatic species [29] and longer for *O. volvulus*. More recent work

has identified the *Wolbachia* endosymbiont of many filarial nematodes as a target for chemotherapy, with several studies in animal models and in human populations showing that treatment with doxycycline leads to clearance of the *Wolbachia* and the subsequent death of the adult worm [30, 31].

As referred to above, many of the compounds currently used for the treatment of helminth infections in humans derive from veterinary medicine, an observation that ought to strike a note of caution. In veterinary practice, anthelmintic resistance is now widespread, particularly amongst parasites of small ruminants such as *Haemonchus contortus*, a close relative of the human hookworms, and *Teladorsagia circumcincta* [32]. As highlighted by Hotez and colleagues [23], control of the six most common helminth infections in over 1 billion people worldwide relies on only four drugs, two benzimidazole compounds and ivermectin (for nematodes) and praziquantel (for schistosomes). While this situation is testament to the safety and efficacy of each of these drugs in mass drug administration programs, it also emphasizes the pressing need for additional compounds in the arsenal, particularly in the context of drug resistance. For example, there is evidence that resistance to ivermectin has already emerged in parts of Africa where it has been extensively used for the control of onchocerciasis, highlighting the critical nature of the problem [33]. Below, we discuss whether Hsp90 inhibitors could be developed to fill this gap in the chemotherapeutic toolbox for nematode parasites of humans or animals.

Hsp90 differs in free-living and parasitic nematodes

In common with other eukaryotes, filarial worms express relatively high levels of Hsp90, but in contrast to other heat shock proteins, Hsp90 is not significantly upregulated, at either the mRNA or protein level, upon temperature shift from 28^{o}C to 37^{o}C (mimicking the vector to mammalian transition) or upon heat shock [34, 35]. What then might be the function of Hsp90 in these organisms? Hsp90 is clearly essential in filarial nematodes and the high level of constitutive expression perhaps facilitates survival in the potentially hostile environment of a mammalian host. By analogy with other eukaryotes, we assume that Hsp90 chaperones a number of important client proteins in *Brugia* that are essential for viability and the *Brugia malayi* genome (http://parasite.wormbase.org) contains homologs of many known client proteins and co-chaperones identified in other organisms [36].

Filarial nematodes are difficult to work with, requiring both mosquito and mammalian hosts for maintenance, as it is not possible to grow them *in vitro*. Further, for most parasitic nematodes, genetic manipulation is in its infancy [37] and RNAi produces variable results [38]. For these reasons, we turned to the free-living nematode *Caenorhabditis elegans*, to better understand the function of Hsp90. *C. elegans* is extensively used as a model for defining gene function in parasitic nematodes and many previous studies have demonstrated successful complementation of *C. elegans* mutants with genes from parasitic nematodes [39, 40]. As might be expected, Hsp90 (DAF-21 in *C. elegans*) is essential as demonstrated by the phenotype of a null mutant, which arrests growth at the L2-L3 stage [41]. It was something of a surprise, therefore, when it was reported that *C. elegans* DAF-21 differed from most other eukaryotic Hsp90s, in that it was not susceptible to the N-terminal Hsp90 inhibitor, GA. Growth of *C. elegans* on plates containing GA or, indeed, on *Streptomyces hygroscopicus* (the organism which produces GA) as the sole food source, failed to produce a phenotype [42].

In addition to growing normally on GA, *C. elegans* DAF-21 was also unable to bind GA covalently linked to a solid support [42]. In these experiments DAF-21 originated from either a worm lysate or from *in vitro* translation products. By substituting the N or C terminal of the worm molecule with chicken Hsp90, it was shown that the deficiency lay in the N-terminal region of DAF-21. Using a similar assay in which worm lysate is mixed with GA beads, we confirmed these findings with *C. elegans*, while demonstrating that Hsp90 from the filarial nematode *Brugia pahangi* bound to GA beads [35]. Furthermore, in a fluorescence polarization assay, which also utilized worm lysate, we could detect no significant binding of labeled GA to *C. elegans* extract, while Hsp90 from *Brugia* extract bound with similar affinity to that of a breast cancer cell line. In addition, there was no significant difference in GA binding to extracts of wild-type *C. elegans*, or to worms in which levels of DAF-21 were reduced by ~40% by RNAi [43]. Given the degree of similarity (92%) between the two nematode Hsp90 sequences, these data are hard to explain on the basis of sequence diversity alone. However, Hsp90 does not function in isolation and in worm lysate is likely to be complexed with a multitude of co-chaperones and client proteins that could modulate its ability to bind inhibitors.

Further evidence that the Hsp90 interactome may be key to function, and may differ significantly between parasitic filarial nematodes and the free-living *C. elegans*, came from additional studies in

which we attempted to rescue the phenotype of a *C. elegans daf-21* mutant with *B. pahangi hsp90*. Worms were injected with a *Brugia hsp90* genomic construct under the control of the *daf-21* promoter and 3'UTR, in an attempt to mimic expression of the endogenous gene. Despite showing that the *Brugia* Hsp90 protein was expressed in transfected *C. elegans* and able to bind to GA in a pull-down assay, we were unable to rescue the mutant phenotype [44]. In the same study, we also attempted to rescue the *C. elegans daf-21 (RNAi*) phenotype with transgenes from *B. pahangi* or *H. contortus*, the latter being a clade V nematode and more closely related to *C. elegans*. No rescue was obtained with the *Brugia* transgene while the *H. contortus* transgene provided partial rescue. Although *Brugia* and *C. elegans* belong to different clades, there are several examples in the literature of successful rescue of *C. elegans* mutants with the orthologous gene from *Brugia* spp., even when the degree of conservation is significantly less than that of the respective Hsp90s [45]. While failure to rescue the mutant phenotype may reflect differences in the nature of the client proteins chaperoned by Hsp90 in *Brugia* and *C. elegans*, a diversity of additional factors are known to contribute to the regulation of Hsp90 function [46]. These include variation in co-chaperone usage and differences in post-translational modification of Hsp90 and of its co-chaperones, such as phosphorylation, acetylation or sumoylation, each of which can modulate activity of the complex [47].

In additional studies, we sought to determine whether the lack of binding of DAF-21 to GA was specific to *C. elegans* or shared with other nematode species. We analyzed twenty-four different species of parasitic and free-living nematode belonging to each of the five clades of nematode [48]. That study produced an interesting spectrum of binding activity: Hsp90 from all free-living species tested failed to bind GA in a pull down. Many parasitic nematodes that dwell in the gastro-intestinal tract have free-living larval stages in the environment and such species (*Haemonchus*, *Teladorsgia* etc.) also failed to bind GA. In contrast, those species where the free-living stage in the environment is protected within an egg (e.g. *Ascaris*, *Toxocara*) all bound GA, as did Hsp90 from obligate parasites (no free-living stages), such as filarial nematodes and *Trichinella*. These data support the adaptive evolution hypothesis first proposed by David and colleagues [42]; free-living stages of nematodes in the environment may encounter inhibitors such as GA, which is produced by a soil-dwelling *Streptomyces*, and thus may be under selective pressure to evolve a GA-resistant Hsp90. In contrast, parasites in which the free-living stage is enclosed within a protective egg, or obligate parasites such as

filarial nematodes, which never encounter the environment, are under no such pressure [49]. We next attempted to determine whether there was evidence of adaptive evolution in nematode *hsp90* sequences in support of this hypothesis. While we could identify amino acid residues showing evidence of adaptive evolution along three separate lineages, it was not possible within the fifteen nematode species analyzed to identify specific residues that correlated with GA susceptibility or resistance [48]. Additional studies comparing structural features of nematode Hsp90 will be required to further illuminate the significance of the variation in amino acid sequences in species with different GA binding properties.

In contrast to our studies on nematodes, work on the yeast *Humicola fuscoatra* has highlighted a single amino acid residue associated with decreased binding of the Hsp90 inhibitor, RAD. As referred to previously, many Hsp90 inhibitors are natural products produced by a range of soil bacteria or fungi. *H. fuscoatra* produces the N-terminal inhibitor RAD and provides an excellent example of an adaptation to survive self-toxicity [50]. Here a single amino acid residue (L34 to I) results in a RAD-resistant phenotype, although sensitivity to GA or ATPase activity is not significantly affected. Introduction of this mutation into *Sachromyces cerevisiae* results in a lowered affinity for RAD. By structural analysis it was shown that the L to I change enlarges the hydrophobic pocket, allowing the binding of additional H_2O molecules, with the resulting increase in hydration disfavoring binding of the drug.

Within the nematodes, the presence of GA binding and non-binding species raises the important question as to whether organisms that are susceptible to N-terminal Hsp90 inhibitors could develop resistance to these compounds, if used as drugs. It is difficult to address this question in the absence of a full explanation of the molecular basis of GA binding diversity. However, in previous studies, David *et al* [42] expressed the GA-resistant *C. elegans hsp90* in mammalian cells and found that while it formed heterodimers with the endogenous Hsp90, it did not confer resistance to GA.

Much of the difference between *Brugia* and *C. elegans* may lie in the nature of the Hsp90 complex or in the client proteins chaperoned by Hsp90 and our ongoing studies have produced some support for this hypothesis. Using PU-H71 pull-down assays, as described above, preliminary analysis has

demonstrated that the *Brugia* Hsp90 complex bears a striking resemblance to that from tumor cells, containing specific activator co-chaperones and client proteins indicative of a high affinity Hsp90 complex (Rodina et al, unpublished data). For example, the integrin signaling, cell adherence and glucose metabolism pathways were all represented in the *Brugia* pull-down but were absent from the equivalent *C. elegans* pull-down. The similarity in the Hsp90 complex in *Brugia* and tumor cells is intriguing. Filarial worms are extraordinarily well adapted to life in the mammalian host; many species are specific to humans, they are long-lived and have evolved multiple mechanisms to modulate the immune response of their hosts and ensure their own survival [51]. One interpretation of these data is that obligate parasites, such as *Brugia*, may have an increased reliance on Hsp90 for survival in the potentially hostile environment of the host, whereas free-living nematodes such as *C. elegans* may have a low affinity Hsp90, perhaps comparable to the differences in Hsp90 between malignant and non-malignant cells. On the one hand, the constitutive expression of Hsp90 and the apparent existence of a high activity Hsp90 complex in filarial worms may aid their survival, but, on the other hand, it could render them more susceptible to the effects of Hsp90 inhibition.

The pros and cons of Hsp90 inhibitors for the control of parasitic nematodes

Many drugs with nematocidal activity, with the exception of the benzimidazole compounds and those targeting the filarial *Wolbachia* endosymbiont, affect the neuro-muscular junction in nematodes resulting in paralysis of the worm and subsequent expulsion from the body (reviewed in [52]). The mode of action of Hsp90 inhibitors will be quite different, as the inhibition of Hsp90 function results in the destabilization of a myriad of client proteins. Thus, it could be argued, a parasite may be less likely to develop resistance to an inhibitor that affects multiple key pathways, than to a drug that exerts a strong selective pressure on a single molecular target. However, as Hsp90 is expressed by host as well as by the parasite, ensuring that an inhibitor preferentially targets parasite Hsp90 provides a challenge. This can be done by medicinal chemistry approaches, exploiting the subtle differences in Hsp90 sequence between host and parasite. Recent work on *T. brucei* Hsp83 [18] and on Hsp90 from the malaria parasite, *P. falciparum*, [17] provides good examples of this approach. However, as discussed previously, at least for some parasitic nematodes, differences in the function of Hsp90 between parasite and host may provide a chink in the armor suitable for exploitation.

In early studies on filarial worms, we demonstrated that inhibition of Hsp90 by GA was sufficient to kill adult *B. pahangi* [35]. The effect of these compounds on the worms is first manifest by a reduction in Mf output, followed by the irreversible cessation of Mf production. Death of the adult worm follows. However, while the inability to produce Mf might accelerate worm death, male worms also die when Hsp90 is inhibited. Many pathogenic filarial worms, including *Brugia* spp., carry a *Wolbachia* endosymbiont, which contains an ortholog of the bacterial Hsp90, HtpG [53]. To investigate whether the sensitivity of *Brugia* parasites to GA was related to the presence of the endosymbiont, we exposed the *Wolbachia*-free filarial species, *Acanthocheilonema viteae* to GA. However, the susceptibility of these worms to GA was very similar to that of *Brugia*, suggesting that the presence of *Wolbachia* has little impact upon sensitivity to GA and that the major effect of the drug is on worm Hsp90 [35].

A number of other chemical scaffolds including purine-scaffold and isoxazole-based compounds have now been tested against filarial worms with various levels of activity [43, 54]. For example, our recent work demonstrated that filarial worms were killed by *in vitro* exposure to 25.0 nM NVP-AUY922 and showed that this compound was also active *in vivo* against adult worms [54]. While our studies have focused largely on *B. pahangi*, we, and others [21], have shown similar effects on the human pathogen *B. malayi* and would predict that the related filarial parasite of the dog, the heartworm *Dirofilaria immitis*, is equally susceptible. Heartworm is an important pathogen in North America and most owned animals in endemic areas are on prophylactic therapy with ivermectin or related macrocyclic lactones. Heartworm control represents a major area for the pharmaceutical industry, given that treatment costs are in the order of $75-100 per animal per annum and that there are approximately 80 million potential hosts in the USA (quoted in [55]). Moreover, recent data demonstrate that resistance to ivermectin has been detected in *D. immitis* from dogs in the Mississippi river delta [56], again highlighting the need for alternative therapies.

Some of the most interesting new approaches for tumor therapy are based on the observation that Hsp90 inhibitors accumulate in tumor cells and recent experimental studies have used this technology to transport imaging reagents into tumors [56[57]. This approach, which is similar in principle to that by which antibodies are used to target drugs to tumors, shows significant potential for the development of new therapeutics. Using such chimeric conjugates, drugs are targeted to and concentrate within

tumors and several such conjugates are undergoing clinical trials at present. Whether a similar methodology would be successful with parasitic organisms and might allow the selective accumulation of drug within a parasite deserves further study.

Hsp90 and drug resistance

As referred to in this review, a major obstacle in the control of many parasitic diseases is the inexorable rise and spread of drug resistance. While this is particularly important in human pathogens such as the malaria parasite, *P. falciparum* [58] it is also an increasing problem in veterinary pathogens [59] and in various vectors of disease, such as mosquitoes [60]. In this respect, Hsp90 may be worthy of additional study, as in some organisms it facilitates the development of drug resistance. This concept runs counter to the prevailing view of drug resistance in many pathogens, where most effort is focused on the search for genetic mutations that underlie this phenotype. While the availability of multiple helminth genome sequences (http://parasite.wormbase.org) and the analysis of genetic crosses between susceptible and resistant worms [61] is facilitating the identification of regions of the genome associated with resistance, much remains to be learned. The role of Hsp90 in resistance relates to its ability to buffer mutations, as was first demonstrated in *Drosophila melanogaster*, where inhibition of Hsp90 function allowed the appearance of a range of phenotypic abnormalities. It was proposed that high levels of Hsp90 buffered existing cryptic variation, which could then be expressed when Hsp90 was inhibited [62]. These observations were shown to have important practical applications in pathogens, such as fungi where drug resistance is an increasing problem [63]. Chemical inhibition of Hsp90 function, or reduction in Hsp90 levels by genetic means, reversed azole resistance in a range of fungal organisms, including pathogenic fungi [64]. Anti-fungal agents induce a cellular stress response, which is stabilized by Hsp90, allowing the emergence of resistant isolates, which are then compromised when Hsp90 function is reduced. These studies provide a rare, and encouraging, example of the reversal of drug resistance in a pathogen. More recently, a similar phenomenon was described in hormone-dependent breast tumor cells where the ability of Hsp90 to buffer mutations facilitates the development of resistance. Blocking Hsp90 function with very low doses of inhibitor reversed the resistant phenotype, offering a novel approach to combating the drug resistance observed in many tumor types following chemotherapy [65]. The observation that Hsp90 facilitates the drug resistance phenotype in organisms as divergent as fungi and tumor cells, suggests that this phenomenon may have broader

application. In preliminary studies, we assessed the impact of reducing levels of DAF-21 by RNAi in ivermectin-resistant isolates of *C. elegans*, but detected no modulation of resistance (Him, Gillan and Devaney, unpublished data). However, this area should be revisited to rule out a possible role for Hsp90 in facilitating the development of anthelmintic resistance in nematodes.

In conclusion, our own studies and those of many others have highlighted the potential of Hsp90 as a target for parasite chemotherapy. Many different parasite species, both protozoan and helminth, are susceptible to inhibition of Hsp90 and significant progress has been made in recent years in identifying inhibitors with a degree of parasite specificity. However much remains to be learned before any of these compounds progress to human trials. Many pharmaceutical companies have large libraries of Hsp90 inhibitors, which could be repurposed for the control of neglected tropical diseases, perhaps providing a short cut to the development of new therapeutics. As referred to above, Hsp90 is a protein with apparently contradictory functions: while it is a recognized target in tumors cells, it also has a completely different role in drug resistance. Whether these findings may be indicative of a more general role for Hsp90 in the evolution of drug resistance in other eukaryotic pathogens deserves further study.

Conflicts of interest: The authors declare no conflicts of interest

Acknowledgements: Work in the authors' laboratory was supported by grants from the BBSRC (BB/E013473/1) and the Wellcome Trust (076734/Z/05/Z). We would like to thank Drs Jane Kinnaird, Roz Laing and Alan Winter for helpful comments on the manuscript.

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